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(57) Abstract

The present invention relates to methods and reagents for extending the life-span, e.g., the number of mitotic divisions, of a cell. In general, the subject method relies on the ectopic expression of the telomerase catalytic subunit EST2, or a bioactive fragment thereof. The subject method is useful both *in vivo*, *ex vivo* and *in situ*.

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Extension of Cellular Lifespan, Methods and Reagents

Background of the Invention

The linear chromosomes of eukaryotic cells offer the biological advantages of rapid recombination, assortment, and genetic diversification. However, linear DNA is inherently more unstable than circular forms. To address this difficulty, the eukaryotic chromosome has evolved to include a DNA-protein structure, the telomere, which caps chromosome ends and protects them from degradation and end-to-end fusion (Blackburn (1984) Annu Rev Biochem 53:163-194; Blackburn (1991) Nature 350:569-573; Zakian (1995) Science 270:1601-1607).

The DNA component of telomeres consists of tandem repeats of guanine-rich sequences tha re essential for telomere function (Blackburn, supra; Zakian, supra). These repeats are replicated by conventional DNA polymerases and by a specialized enzyme, telomerase (Greider (1995) "Telomerase Biochemistry and Regulation" In: Telomeres, E.H. Blackburn and C.W. Greider, Eds. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp.35-68), first identified in the ciliate Tetrahymena (Greider and Blackburn (1985) Cell 43:405-413). The telomerase enzyme is essential for complete replication of telomeric DNA because the cellular DNA-dependent DNA polymerases ar unable to replicate the ultimate ends of the telomeres due to their requirement for a 5' RNA primer and their unidirectional mode of synthesis. Removal of the most terminal RNA primer following priming of DNA synthesis leaves a gap that cannot be replicated by these polymerases (Olovnikov (1971) Dokl. Akad. Nauk SSSR 201:1496-1499; Watson (1972) Nat New Biol 239:197-201). Telomerase surmounts this problem by do novo addition of single-stranded telomeric DNA to the ends of chromosomes (Greider and Blackburn (1985) supra; Greider and Blackburn (1989) Nature 337:331-337; Yu, et al. (1990) Nature 344:126-132; Greider (1995) supra).

The telomerase enzymes that have been charcterized to date are RNA-dependent DNA polymerases that synthesize the telomeric DNA repeats by using an RNA template that exists as a subunit of the telomerase holoenzyme (Greider (1995), supra). The genes specifying the RNA subunits of telomerases have been cloned from a wide variety of species, including humans (Feng, et al. (1995) Science 269:1236-1241; Greider (1995), supra), and have been shown in several instances to be essential for telomerase function in vivo (Yu, et al. supra; Yu and Blackburn (1991) Cell 67:823-832; Singer and Gottschling (1994) Science 266:404-409; Cohn and Blackburn (1995) Science 269:396-400; McEachern and Blackburn (1995) Nature 376:403-409). In addition, three proteins have been identified to date that rae associated with telomerase activity. P80 and p95 were purified from the ciliate Tetrahymena (Collins, et al. (1995) Cell 81:677-686), and the gene encoding a mammalian homolog of p80, TP1/TLP1, has also been cloned (Harrington, et al. (1997) Science 275:973-977; Nakayama, et al. (1997) Cell 88:875-884). The specific mechanism by which these proteins participate in telomerase function has not been defined.

Most recently, two related proteins, Est2p from the yeast Saccharomyces cerevisiae, and p123 from the ciliate Euplotes aediculatus, were identified as the catalytic subunits of telomerase in their respective species (Counter, et al. (1997) PNAS USA 94:9202-9207; Lingner, et al. (1997) Science 276:561-567). EST2 was first identified as a gene required for telomere maintenance in yeast (Lendvay, et al. (1996) Genetics 144:1399-1412) and is essential for telomerase activity (Counter, et al. supra; Lingner, et al. supra). Both the yeast and Euplotes proteins harbor several sequence motifs that are hallmarks of the catlaytic regions of reverse transcriptases; substitution of several such residues in Est2p abolishes telomerase activity (Counter, et al. supra; Lingner, et al. supra). The mammalian homolog of these telomerase subunits has not yet been reported.

As might be expected from the known enzymatic properties of telomerase, perturbing the function of this enzyme in the ciliate Tetrahymena, through the overexpression of an inactive form of the telomerase RNA, or in yeast, through the mutation of genes encoding either the catalytic protein or template RNA subunit, leads to progressive telomere shortening as cells pass through successive cycles of replication (Yu, et al. supra; Singer and Gottschling supra; McEachern and Blackburn supra; Lendvay, et al. supra; Counter, et al. supra; Lingner, et al. supra). This loss of telomeric DNA is ultimately lethal if it is not overcome. The lethality seems to be triggered when telomeres have been truncated below a critical threshold level. Hence, in the absence of compoensating mechanisms, yeast cell lineages that lack telomerase activity have a lifespan dictated by the lenths of their telomeres.

In humans, telomerase activity is readily detectable in germline cells and in certain stem cell compartments. However, enzyme activity is not dtectable in most somatic cell lineages (Harley, et al. (1994) Cold Spring Harbor Symp. Quant. Biol. 59:307-315; Kim, et al. (1994) Science 266:2011-2015; Broccoli, et al. (1995) PNAS USA 92:9082-9086; Counter, et al. (1995) Blood 85:2315-2320; Hiyama, et al. (1995) J Immunol 155:3711-3715). Consistent with this, telomeres of most types of human somatic cells shorten with increasing organismic age and with repeated passaging in culture, similar to the situation seen in protozoan and yeast cells that have been deprived experimentally of a functional telomerase enzyme (Harley, et al. (1990) Nature 345:458-460; Hastie, et al. (1990) Nature 346:866-868). Eventually, the proliferation of cultured human cells will halt at a point termed senescence (Hayflick and Moorhead (1961) Exp Cell Res 25:585-621; Goldstein (1990) Science 249:1129-1133), apparently before the telomeres of these cells have become critically short.

Cultured normal human cells can circumvent senscence and thereby continue to proliferate when transformed by a variety of agents. In such cultures, telomere shortening continues until a subsequent point is reached that is termed crisis, where telomeres have become

extremely short (Counter, et al. (1992) EMBO J 11:1921-1929; Counter, et al. (1994a) J Virol 68:3410-3414; Shay, et al. (1993) Oncogene 8:1407-1413; Klingehutz, et al. (1994)). Crisis, perhaps best described in SV40-transformed cells, is characterized by karyotypic instability, particularly the types of instability observed in chromosomes lacking functional telomeres, and by significant levels of cell death (Sack (1981) In Vitro 17:1-19). The crisis phenotype is reminiscent of that observed in yeast and Tetrahymena cells in which telomerase function hasbeen experimentally perturbed.

The simplest interpretation of these data is that the lifespan of telomerase-negative human cells, like that of their yeast and ciliate counterparts, is ultimately limited by the length of telomeres. Rare human cells that have acquired the ability to grow indefinitely emerge from crisis populations with a frequency of 10^{-6}-10^{-7} (Huschtscha and Holliday (1983) J Cell Sci 63:77-99; Shay and Wright (1989) Exp Cell Res 184:109-118). This implies that amutational event is required to confer the immortal phenotype on these cells. The immortal cells that escape crisis are characterized by readily detectable levels of telomerase activity and by stable telomeres (Counter, et al. (1992) supra; Counter, et al. (1994a) supra; Shay, et al. (1995) Mol Cell Biol 15:425-432; Whitaker, et al. (1995) Oncogene 11:971-976; Gollahon and Shay (1996) Oncogene 12:715-725; Klingelhutz, et al. (1996) Nature 380:79-82). This suggests that activation of telomerase can overcome the limitations imposed by telomere length of the lifespan of cell lineages.

Activation of telomerase also appears to be a major step in the progression of human cancers. Unlike normal human cells, cancer cells can be established as permanent cell lines and thus are presumed to have undergone immportalization during the process of tumorigenesis. Moreover, telomerase activity is readily detected in the great majority of human tumor smaples analyzed to date (Counter, et al. (1994b) PNAS USA 91:2900-2904; Kim, et al. 1994 supra); Shay and Bacchetti (1997) Eur J Cancer 33:787-791).

Taken together, these various observations have been incorporated into a model that proposes that the limitation on prolonged cell replication imposed by telomere shortening serves as an important antineoplastic mechanism used by the body to block the expansion of pre-cancerous cell clones. According to such a model, tumor cells transcend the crisis barrier and emerge as immortalized cell populations by activating previously unexpressed telomerase, enabling them to restore and maintain the integrity of their telomeres (Counter, et al. (1992) supra; Counter, et al. (1994a) supra; Harley, et al. (1994) supra).

A major question provoked by this model is the mechanism used to resurrect telomerase expression during tumor progression. Expression of the telomerase-associated protein

TP1/TLP1 does not reflect the level of telomerase activity (Harrington, et al. supra; Nakayama, et al. supra). It is also clear that the levels of the human telomerase RNA component, hTR, cannot completely explain the regulation of telomerase activity. Although the levels of hTR and its mouse counterpart, mTR, increase with tumor progression (Feng, et al. (1995) Science 269:1236-1241; Blasco, et al. (1996) Nat Genet 12:200-204; Broccoli, et al. (1996) Mol Cell Biol 16:3765-3772; Soder, et al. (1997) Oncogene 14:1013-1021), the amounts of these transcripts do not always correlate with enzymatic activity. Indeed, hTR or mtr transcript levels can be significantly higher in telomerase-negative cells and tissues than in telomerase-positive cancer cells (Avilion, et al. (1996) Cancer Res 56:645-650; Bestilny, et al. (1996) Cancer Res 56:3796-3802; Blasco, et al. supra). Similarly, even though telomerase levels increase 100- to 2000-fold during the immortalization of huma n cells, the level of hTR message increases, at most, two-fold (Avilion, et al. supra). Therefore, depression of the hTR and TP1 subunits cannot easily be invoked to explain the appearance of telomerase activity in the great majority of human tumor samples. Thus far, the rate-limiting step in telomerase activation has remained elusive.

Summary of the Invention

One aspect of the present invention relates to methods and reagents for extending the life-span, e.g., the number of mitotic divisions, of a cell. In general, the subject method relies on the activation of a telomerase activity, such as by ectopic expression of the telomerase catalytic subunit EST2, or a bioactive fragment thereof, or the ectopic expression of *myc*, or a bioactive fragment thereof, or by contacting the cell with an agent (such as a small organic molecule) which activates expression of EST2 or *myc* or relieves an inhibitory signal (antagonism) of *myc*. By "ectopic expression", it is meant that a cell is caused to express, e.g., by expression of a heterologous or endogenous gene or by transcellular uptake of a protein or inhibition of degradation of the EST2 or *myc* protein, a higher than normal level of EST2 or *myc* than the cell normally would for the particular starting phenotype. The subject method is useful both *in vivo*, *ex vivo* and *in situ*. Exemplary uses include, merely to illustrate, the extension of stem cell or progenitor cell cultures or implants, the extension of skin or other epithelial cell cultures or grafts, the expansion of mesenchymal cell cultures or grafts, and the expansion of chondrocyte or osteocyte cultures or grafts. Exemplary stem and progenitor cells which can be extended by the subject method include neuronal, hematopoietic, epithelial, pancreatic, hepatic, chondrocytic and osteocytic stem and progenitor cells. The subject method can be used for wound healing and other tissue repair, as well as cosemetic uses. It can be applied for prolonging the lifespan of a culture of normal cells or tissue being used to secrete therapeutic or other commercially significant proteins and products.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Brief Description of the Drawings

Figure 1. HEST2 encodes a human homolog of Est2p and p123. Alignment of the predicted amino acid sequence of HEST2 with the yeast Est2p and Euplotes p123 homologs. Amino residues within shaded and closed blocks are indentical between at least two proteins. Indentical amino acids within the RT motifs are in closed boxes, an example of a telomerase-specific motif in an outlined shaded box, and all identical amino acids in shaded boxes. RT motifs are extended in some cases to include other adjacent invariant or conserved amino acids. The sequence of the expressed tag AA281296 is underlined.

Figure 2. Alignment of RT motifs 1-6 of telomerase subunits HEST2, p123 and Est2p with S Cerevisiae group II intron-encoded RTs a2-Sc and a1-Sc. The consensus sequence of each RT motif is shon (h=hydrophobic, p=small polar, c=charged). Amino acids that are invariant among the telomerases and the RT consensus are in shaded boxes. Open boxes identify highly conserved residues unique to either telomerases or to nontelomerase RTs. Astericks denote amino acids essential for polymerase catalytic function.

Figure 3. *Myc* activation of telomerase in HMEC cells. Primary HMEC cells at passage 12 were infected with empty vector (lanes 1-5), E6 (lanes 6-10), c-*myc* (lanes 11-15) or cdc25A

(lanes 16-20) viruses. Two breast cancer cell lines BT549 (lanes 21-25) and T47D (lanes 26-30) were included for comparison. The cells were lysed and TRAP assays were performed using extract corresponding to 10,000 cells (lanes 2, 6, 7, 11,12, 17, 21, 22, 26 and 27), 1,000 cells (lanes 3, 8, 13, 18, 23 and 28), 100 cells (lanes 4, 9, 14, 19, 24 and 29) or 10 cells (lanes 5, 10, 15, 20, 25 and 30). Telomerase activity was shown to be sensitive to RNase by the addition of RNase A prior to the telomerase assay ("-", without RNase A; "+", with RNase A). To rule out the presence of inhibitors in apparently negative lysates, lanes labelled "Mix" (lanes 1 and 16) are assays containing lysate from 10,000 of the indicated cells mixed with lysate from 10,000 positive (c-*myc*-expressing) cells .

Figure 4. *Myc* activaton of telomerase in IMR90 fibroblasts. IMR90 cells at passage 14 were infected with empty vector (lanes 1-5), c-*myc* (lanes 6-10) and E6 (lanes 11-15) viruses. HT1080 cells (lanes 15-20) were included for comparison. TRAP assays contained 10,000 cells (lanes 2, 6, 7, 12, 16 and 17), 1,000 cells (lanes 3, 8, 13 and 18), 100 cells (lanes 4, 9, 14 and 19) or 10 cells (lanes 5, 10, 15 and 20). Telomerase activity was shown to be sensitive to RNase by the addition of RNase A prior to extention reaction ("-", without RNase A; "+", with RNase A). "Mix" lanes (1 and 11) are assays containing lysate from 10,000 of the indicated cells mixed with lysate from 10,000 positive (c-*myc*-expressing) cells.

Figure 5. E6 increases c-*myc* protein level in HMEC. **A.** Levels of *myc* protein were determined by western blotting with a polyclonal *myc* antibody. Cell lysates from E6 (lane 1) and vector (lane 2) infected IMR90 cells and lysates from c-*myc* (lane 3), E6 (lane 4) and vector (lane 5) infected HMEC cells were analyzed. Tumor cell lines, HT1080 (lane 6), HBL100 (Lane 7), BT549 (lane 8) and T47D (lane 9), were included for comparison. The expression of TFIIB was used to normalize loading. **B.** Total RNA prepared in parallel with the protein extracts used in **A.** was used in northern blots to determine *myc* mRNA levels. Equal quantities of total RNA, as indicated, were probed with a human c-*myc* cDNA.

Figure 6. Extention of telomere length and cellular lifespan by telomerase activation. **A.** Total RNA was prepared from normal HMEC and from HMEC that had been infected with a *myc* retrovirus. hEST2 transcript was visualized in equal quantities of RNA (10 μg) using a probe derived from the hEST2 cDNA. **B.** HMEC and IMR90 cells were infected with either empty vector (lanes 1-5 and 11-15) or hEST2 (lanes 6-10 and 16-20) viruses. TRAP assays were performed using lysate equivalent to 10,000 cells (lanes 2, 6, 7, 12, 16 and17), 1,000 cells (lanes 3, 8, 13 and 18), 100 cells (lanes 4, 9, 14 and 19) or 10 cells (lanes 5, 10, 15 and 20). Telomerase activity was shown to be sensitive to RNase by the addition of RNase A prior to assay ("-", without RNase A; "+", with RNase A). To rule out the presence of inhibitors in apparently negative lysates, lanes labelled "Mix" (lanes 1 and 16) are assays containing lysate

from 10,000 of the indicated cells mixed with lysate from 10,000 positive (HT1080) cells. **C.** Genomic DNA from early passage HMEC (passage 12, lane 1), late passage HMEC (passage 22, lane 2), HMEC/hEST2 (cells infected at passage 12 with hEST2 and subsequently cultured for 10 additional passages, lane 3) and HMEC/vector (cells infected at passage 12 with empty vector and subsequently cultured for 10 additional passages, lane 4) were digested with *Rsa* I and *Hinf* I. Fragments were separated on a 0.8% agarose gel, and telomeric restriction fragments were visualized using a ^{32}P-labled human telomeric sequence $(TTAGGG)_3$ as a probe. **D.** HMEC cells were transduced at passage 12 with either empty vector, c-*Myc* or hEST2 retroviruses (as indicated). These cells were continuously subcultured at a density of 4-5x10^5 cells per 100 cm^2 once per week. After 12 passages following transduction, vector-infected cells could no longer be subcultured at this frequency and adopted a classic senescent phenotype. In contrast, cells expressing *myc* and hEST2 continue to proliferate and showed a virtual absence of sensescent cells in the population.

Figure 6. Illustrates a MarxII vector including the coding sequence for hEST2. The long terminal repeats (LTRs) include, though not shown, recombinase sites such that, upon treatment of a cell in which the MarxII-hEST2 vector is integrated, the proviral vector including the hEST2 coding sequence is excised.

Detailed Description of the Invention

Normal mammalian diploid cells placed in culture have a finite proliferative life-span and enter a nondividing state termed senescence, which is characterized by altered gene expression (Hayflick et al. (1961) Exp. Cell Res. 25:585; Wright et al. (1989) Mol. Cell. Biol. 9:3088; Goldstein, (1990) Science 249:112; Campisi, (1996) Cell 84:497; Campisi (1997) Eur. J. Cancer 33:703; Faragher et al. (1997) Drug Discovery Today 2:64). Replicative senescence is dependent upon cumulative cell divisions and not chronologic or metabolic time, indicating that proliferation is limited by a "mitotic clock" (Dell'Orco et al. (1973) Exp. Cell Res. 77:356; Hadey et al. (1978) J. Cell. Physiol. 97:509). The reduction in proliferative capacity of cells from old donors and patients with premature aging syndromes (Martin et al. (1970) Lab. Invest 23:86; Schneider et al. (1976) PNAS 73:3584; Schneider et al. (1972) Proc. Soc. Exp. Biol. Med. 141:1092; Elmore et al. (1976) Cell Physiol. 87:229), and the accumulation in vivo of senescent cells with altered patterns of gene expression (Stanulis-Praeger et al. (1987) Mech. Ageing Dev. 38:1; and Dimri et al. (1995) PNAS 92:9363), implicate cellular senescence in aging and age-related pathologies ((Hayflick et al. (1961) Exp. Cell Res. 25:585; Wright et al. (1989) Mol.

Cell. Biol. 9:3088; Goldstein, (1990) Science 249:112; Campisi, (1996) Cell 84:497; Campisi (1997) Eur. J. Cancer 33:703; Faragher et al. (1997) Drug Discovery Today 2:64).

Telomere loss is thought to control entry into senescence. Human telomeres consist of repeats of the sequence TTAGGG/CCCTAA at chromosome ends; these repeats are synthesized by the ribonucleoprotein enzyme telomerase. Telomerase is active in germline cells and, in humans, telomeres in these cells are maintained at about 15 kilobase pairs (kbp). In contrast, telomerase is not expressed in most human somatic tissues, and telomere length is significantly shorter. The telomere hypothesis of cellular aging proposes that cells become senescent when progressive telomere shortening during each division produces a threshold telomere length.

The human telomerase reverse transcriptase subunit (hTRT) has been cloned. See Nakamura et al., (1997) Science 277:955; Meyerson et al., (1997) Cell 90:78; and Kilian et al., (1997) Hum. Mol. Genet. 6:2011. It has recently been demonstrated that telomerase activity can be reconstituted by transient expression of hTRT in normal human diploid cells, which express the template RNA component of telomerase (hTR) but do not express hTRT. See, for example, Wang et al. (1998) Genes Dev 12:1769; and Weinrich et al., (1997) Nature Genet. 17:498. This provided the opportunity to manipulate telomere length and test the hypothesis that telomere shortening causes cellular senescence.

The reported results indicate that telomere loss in the absence of telomerase is the intrinsic timing mechanism that controls the number of cell divisions prior to senescence. The long-term effects of exogenous telomerase expression on telomere maintenance and the life-span of these cells remain to be determined in studies of longer duration.

Telomere homeostasis is likely to result from a balance of lengthening and shortening activities. Very low levels of telomerase activity are apparently insufficient to prevent telomere shortening. This is consistent with the observation that stem cells have low but detectable telomerase activity, yet continue to exhibit shortening of their telomeres throughout life. Thus, a threshold level of telomerase activity is likely required for life-span extension.

Cellular senescence is believed to contribute to multiple conditions in the elderly that could in principle be remedied by cell life-span extension in situ. Examples include atrophy of the skin through loss of extracellular matrix homeostasis in dermal fibroblasts; age-related macular degeneration caused by accumulation of lipofuscin and downregulation of a neuronal survival factor in RPE cells; and atherosclerosis caused by loss of proliferative capacity and overexpression of hypertensive and thrombotic factors in endothelial cells.

Extended life-span cells also have potential applications ex vivo. Cloned normal diploid cells could replace established tumor cell lines in studies of biochemical and physiological

aspects of growth and differentiation; long-lived normal human cells could be used for the production of normal or engineered biotechnology products; and expanded populations of normal or genetically engineered rejuvenated cells could be used for autologous or allogeneic cell and gene therapy. Thus the ability to extend cellular life-span, while maintaining the diploid status, growth characteristics, and gene expression pattern typical of young normal cells, has important implications for biological research, the pharmaceutical industry, and medicine.

(i) Overview

One aspect of the present invention relates to methods and reagents for extending the life-span, e.g., the number of mitotic divisions, of a cell. In preferred embodiments, the cells are isolated in culture for at least a portion of the treatment.

In general, the invention provides a method for increasing the proliferative capacity of metazoan cells, preferably mammalian cells, and more preferably normal mammalian cells, by contacting the cell with an agent that activates telomerase activity in cell. In certain embodiments, the subject method relies on the ectopic expression of the telomerase catalytic subunit EST2, or a bioactive fragment thereof. By "ectopic expression", it is meant that a cell is caused to express, e.g., by expression of a heterologous or endogenous gene or by transcellular uptake of a protein, a higher than normal level of EST2 than the cell normally would for the particular starting phenotype.

In other embodiments, the subject method can be carried out by the ectopic expression of an activator of telomerase activity (collectively herein "telomerase activator") such as a *myc* gene product of a papillomavirus E6 protein. In preferred embodiments wherein the ectopic expression of the telomerase or telomerase activator involves a recombinant gene, expression of the gene in the host cell is inducible (or otherwise conditionally regulated) and/or the genetic construct including the gene can be readily removed from thehost cell.

In still other embodiments, the subject method can be carried out by contacting the cell with an agent that inhibits degradation (ubiquitin-dependent or independent) of the EST2 protein or telomerase activator in order to increase the cellular half-life of the protein. For example, the method can utilize an agent which inhibits ubiquitination of to increase the cellular half-life of the protein. For example, the method can utilize an agent which inhibits ubiquitination of *myc* and thereby increases the cellular concentration of *myc*. In preferred embodiments, such agents are small, organic molecules, e.g., having molecular weights of less than 5000 amu (more preferably less than 1000 amu), and which are membrane permeant.

In still other embodiments, cellular prolifeartive capacity can be incrased by contacting the cell with an agent, e.g. a small molecule, which relieves or otherwise inhibits a signal which antagonizes *myc*-induced activation of telomerase activity. For instance, agents can be used which disrupt protein-protein interactions involved in inhibition of *myc* activity by, e.g., *mad-max* heterodimers.

The subject method is useful both *in vivo*, *ex vivo* and *in situ*. Exemplary uses include, merely to illustrate, the extension of stem cell or progenitor cell cultures or implants, the extension of skin or other epithelial cell cultures or grafts, the expansion of mesenchymal cell cultures or grafts, and the expansion of chondrocyte or osteocyte cultures or grafts. Exemplary stem and progenitor cells which can be extended by the subject method include neuronal, hematopoietic, pancreatic, and hepatic stem and progenitor cells.

An important feature of certain preferred embodiments of the subject method is the reversibility of activation of telomerase activity, rather than constitutive activation. For example, where a vector is used to ectopically express an EST2 protein or telomerase activator, the vector can be configured so as to be excisable from the cell. Thus, for *ex vivo* therapies, cells can be treated *ex vivo* with a vector encoding EST2 of a telomerase activator, and prior to implantation, the vector can be excised to inhibit further recombinant expression of the construct *in vivo*. In preferred embodiments, the vector can be excised so as to have little to no heterologous nucleic acid sequences in the host cell.

Another aspect of the present invention relates to *in vitro* preparations of cells which have been treated by the subject method. Such cell compositions can be used, e.g., to generate a medicament for transplantation to an animal.

(ii) Definitions

For convenience, certain terms used herein as defined below.

As used herein, the term "fusion protein" is art recognized and refer to a chimeric protein which is at least initially expressed as single chain protein comprised of amino acid sequences derived from two or more different proteins, e.g., the fusion protein is a gene product of a fusion gene.

The art term "fusion gene" refers to a nucleic acid in which two or more genes are fused resulting in a single open reading frame for coding two or more proteins that as a result of this fusion are joined by one or more peptide bonds.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide, including both exonic and (optionally) intronic sequences. A gene, according to the present invention, can be in the form of a DNA construct which is transcribed or an RNA construct which is directly translatable. An exemplary recombinant gene encoding a subject EST2 protein is represented by SEQ. ID NO: 1.

As used herein, the term "transfection" means the introduction of a heterologous nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein with respect to transfected nucleic acid, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of an EST2 or *myc* polypeptide.

"Expression vector" refers to a replicable nucelic acid construct used to express a gene which encodes the desired protein and which includes a transcriptional unit comprising an assembly of (1) genetic element(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively linked to (2) a sequence encoding a desired protein (e.g. an EST2 or *myc* protein), and (3) as necessary, appropriate transcription and translation initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

In the expression vectors, regulatory elements controlling transcription or translation can be generally derived from mammalian, microbial, viral or insect genes The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. Vectors derived from viruses, such as retroviruses, adenoviruses, and the like, may be employed.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to nucleic acid sequences, such as initiation signals, enhancers, and promoters and the like which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of the EST2 or other telomerase activator gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of one of the naturally-occurring forms of a protein.

As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of a urogenital origin, e.g. renal cells, or cells of a neural origin, e.g. neuronal cells. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well.

"Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a promoter or other transcriptional regulatory sequence is operably linked to a coding sequence if it controls the transcription of the coding sequence.

The terms "EST2 proteins" and "EST2 polypeptides" refer to catalytic subunits of telomerase, preferably of a mammalian telomerase, and even more preferably of a human telomerase. Exemplary EST2 proteins are encoded by the nucleic acid of SEQ ID NO:1, or by a nucleic acid which hybridizes thereto. Thus, the EST2 proteins useful in the subject method can be at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, or even at least 95% identical to the human EST2 of SEQ ID NO:2, or a fragment thereof which reconsitutes a telomerase elongation enzyme in a host cell (such as a human cell). A variety of different techniques are available in the art for assessing the activity of a particular EST2 polypeptide, e.g., which may vary in sequence and/or length relative to SEQ ID NO: 1.

The term "telomerase-activating therapeutic agent" refers to any agent which can be used to activation of telomerase activity in a cell, e.g., a mammalian cell. For example, it includes expression vectors encoding EST2, *myc*, E6 or the like, formulations of such polypeptides, small molecule activators of expression of an endogenous telomerase activator gene, inhibitors of degradation of a telomerase activator, to name but a few.

The term "EST2 therapeutic agent" refers to any telomerase-activating therapeutic agent which can be used to cause ectopic expression of an EST2 polypeptide in a cell. For example, it includes EST2 expression vectors, formulations of EST2 polypeptides, and small molecule activators of expression of an endogenous EST2 gene, to name but a few.

The term "derepresses *myc*" refers to the ability of an agent to overcome an antagonism of *myc*, e.g., it may prevent mad/max inactivation of *myc* and thereby activates *myc*.

The term "progenitor cell" refers to an undifferentiated cell which is capable of proliferation and giving rise to more progenitor cells having the ability to generate a large number of mother cells that can in turn give rise to differentiated, or differentiable daughter cells. As used herein, the term "progenitor cell" is also intended to encompass a cell which is sometimes referred to in the art as a "stem cell". In a preferred embodiment, the term "progenitor cell" refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues.

As used herein the term "substantially pure", with respect to progenitor cells, refers to a population of progenitor cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to progenitor cells making up a total cell population. Recast, the term "substantially pure" refers to a population of progenitor cell of the present invention that contain fewer than about 20%, more preferably fewer than about 10%, most preferably fewer than about 5%, of lineage committed cells in the original unamplified and isolated population prior to subsequent culturing and amplification.

The term "cosmetic preparation" refers to a form of a pharmaceutical preparation which is formulated for topical administration.

As used herein, the term "cellular composition" refers to a preparation of cells, which preparation may include, in addition to the cells, non-cellular components such as cell culture media, e.g. proteins, amino acids, nucleic acids, nucleotides, co-enzyme, anti-oxidants, metals and the like. Furthermore, the cellular composition can have components which do not affect the growth or viability of the cellular component, but which are used to provide the cells in a particular format, e.g., as polymeric matrix for encapsulation or a pharmaceutical preparation.

As used herein the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

(iii) Illustrative Embodiments

(A) Exemplary Telomease Activators

In one embodiment, the subject involves the administration of an expression vector encoding an EST2 polypeptide or other telomerase activator polypeptide.

The isolation of a gene the represents the human homolog, EST2, of the yeast and ciliate genes encoding the telomerase catalytic subunits has recently been reported. See Meyerson, et al. (1997) Cell 90:785; and Nakamura et al. (1997) Science 277:955.

The predicted 127 kDa protein shares extensive sequence similarity with the entire sequences of the Euplotes and yeast telomerase subunits (Figure 1) and extends beyond the amino and carboxyl termini of these proteins. A BLAST search reveals that the probabilities of these smilarieites occurring by chance are 1.3×10^{-18} and 3×10^{-13}, respectively. By way of comparison, the probability of similarity between the yeast and Euplotes telomerases in a protein BLAST search is 6.9×10^{-6}. We have named the hiuman gene hEST2 (human EST2 homolog) to reflect its clear relationship with the yeast gene, the first of these genes to be described. EST2 was named because of the phenotype of Ever Shortening Telomerase catalytic subunit (Counter et al. (1997) *supra*; Lingner et al. (1997)).

Like the yeast and ciliate telomerase proteins, hEST2 is a member of the reverse transcriptase (RT) family of enzymes (Figures 1 and 2). Seven conserved sequence motifs, which define the polymerase domains of these enzymes, are shared among the otherwise highly divergent RT family (Poch et al. (1989) EMBO J 8:3867-3874; Xiong and Eickbush (1990) EMBO J 9:3353-3362). P123 and Est2p share six of these motifs with, most prominently, the a2-Sc enzyme, an RT that is encoded within the second intron of the yeast COX1 gene (Kennell et al. (1993) Cell 133-146). These six motifs, includiung the invariant aspartic acid residues known to be required for telomerase enzymatic function (Counter et al. (1997) *supra*; Lingner et al. *supra*), are found at the appropriate positions of the predicted sequence of hEST2 (Figures 1 and 2). Thus, the proposed human telomerase catalytic subunit, like its yeast and ciliate counterparts, belongs to the RT superfamily of enzymes.

Exemplary human EST coding sequence and protein for use in the subject method is provided at GenBank accession AF018167, AF043739 and AF015950. Exemplary EST constructs are also decribed in PCT application WO98/14593 and Ulaner et al. (1998) Cancer Res 58:4168-72, Counter et L. (1998) Oncogene 161217-22, and Vaziri et al. (1998) Curr Biol 8: 279-82. In a preferred embodiment, the EST construct includes an EST coding sequence which

hybridizes under stringent conditions to SEQ ID No: 1, or a coding sequence set forth in GenBank accession AF018167, AF043739 or AF015950. The EST coding sequence can encode an EST protein, or fragment thereof which retains a telomerase activity, which is is at least, for example, 60, 70 , 80, 85, 90 , 95 or 98 percent identical with a sequence of SEQ ID No. 2 or GenBank accession AF018167, AF043739 and AF015950, or identical with one of the enumerated sequences.

In other illustrative embodiments, telomerase activation can be caused by ectopic expression of a *myc* protein, e.g., c-*myc*. An exemplary human *myc* coding sequence is provided at the SWISS-PROT locus MYC_HUMAN, accession P01106. In a preferred embodiment, the *myc* construct includes an *myc* coding sequence which hybridizes under stringent conditions to a coding sequence set forth in SWISS-PROT locus MYC_HUMAN, accession P01106. The *myc* coding sequence can encode a *myc* protein, or fragment thereof which retains the ability to activate a telomerase activity, which is is at least, for example, 60, 70 , 80, 85, 90 , 95 or 98 percent identical with the protein sequence set forth in SWISS-PROT locus MYC_HUMAN, accession P01106, or identical thereto.

In yet other illustrative embodiments, telomerase activation is accomplished by expression of a papillomavirus E6 protein, preferably an E6 protein from a human papillomavirus (HPV), and more preferably an E6 protein from a high risk HPV (e.g., HPV-16 or -18). It may desirable to use an E6 protein which has been mutated so as to be incapable of effecting p53 degradation. In a preferred embodiment, the E6 construct includes an E6 coding sequence which hybridizes under stringent conditions to a coding sequence set forth in EMBL: locus A06324, accession A06324. The E6 coding sequence can encode an E6 protein, or fragment thereof which retains the ability to activate a telomerase activity, which is is at least, for example, 60, 70 , 80, 85, 90 , 95 or 98 percent identical with the protein sequence set forth in EMBL: locus A06324, accession A06324, or identical thereto

In accordance with the subject method, expression constructs of the subject polypeptides may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively transfecting cells *in vitro* or *in vivo* with a recombinant gene. Approaches include insertion of the subject EST2 or telomerase activator gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors can be used to transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out in vivo. It will be appreciated that because

transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

A preferred approach for introduction of nucleic acid encoding a telomerase activator into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes in vivo, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) Blood 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by nucleic acid encoding, e.g., an EST2 or *myc* polypeptide, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA

88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

In choosing retroviral vectors as a gene delivery system for the subject telomerase activator proteins, it is important to note that a prerequisite for the successful infection of target cells by most retroviruses, and therefore of stable introduction of the recombinant gene, is that the target cells must be dividing. In general, this requirement will not be a hindrance to use of retroviral vectors to deliver the subject gene constructs.

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al. (1989) PNAS 86:9079-9083; Julan et al. (1992) J. Gen Virol 73:3251-3255; and Goud et al. (1983) Virology 163:251-254); or coupling cell surface ligands to the viral env proteins (Neda et al. (1991) J Biol Chem 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the env protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the recombinant gene of the retroviral vector.

Another viral gene delivery system useful in the present invention utilitizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be

advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), and smooth muscle cells (Quantin et al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham (1986) J. Virol. 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) Cell 16:683; Berkner et al., supra; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject telomerase activator constructs is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. Curr. Topics in Micro. and Immunol. (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et al. (1989) J. Virol. 63:3822-3828; and McLaughlin et al. (1989) J. Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470; Tratschin et al. (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al. (1988) Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) J. Virol. 51:611-619; and Flotte et al. (1993) J. Biol. Chem. 268:3781-3790).

Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses. In particular, herpes virus vectors

may provide a unique strategy for persistent expression of the subject telomerase activator proteins in cells of the central nervous system, such as neuronal stem cells, and ocular tissue (Pepose et al. (1994) Invest Ophthalmol Vis Sci 35:2662-2666)

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a the subject proteins in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a gene encoding one of the subject proteins can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al. (1992) No Shinkei Geka 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075). For example, lipofection of neuroglioma cells can be carried out using liposomes tagged with monoclonal antibodies against glioma-associated antigen (Mizuno et al. (1992) Neurol. Med. Chir. 32:873-876).

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as poly-lysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, WO92/19749, and WO92/06180). For example, the subject gene construct can be used to transfect hepatocytic cells in vivo using a soluble polynucleotide carrier comprising an asialoglycoprotein conjugated to a polycation, e.g. poly-lysine (see U.S. Patent 5,166,320). It will also be appreciated that effective delivery of the subject nucleic acid constructs via receptor-mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al. (1993) Science 260-926; Wagner et al. (1992) PNAS 89:7934; and Christiano et al. (1993) PNAS 90:2122).

While the repair of telomers, e.g., by the activation of telomerase activty, can be enough for extending the replicative capacity of a cell, it can be a transforming event (e.g., to cause crisis and emergence of cancer cells), particularly where activation persists. Therefore, in one aspect, the present invention provides a method for increasing the proliferative capacity of cells,

preferably normal cells, which method comprises delivering into the cell a gene construct which can *selectively* and *reversibly* activate telomerase activity in the cell.

In one embodiment, the coding sequence for the telomerase activator is provided as part of a vector which can be partially or completely excised from the host cell is an inducible manner. For instance, the vector can include:

(i) one or more transposition elements for integration of the vector into chromosomal DNA of a eukaryotic host cell;

(ii) a coding sequence of a telomerase activator; and

(iii) excision elements for removing, upon contact of the cell with an excision agent (which activates the excision element) all or at least the portion of an integrated form of the vector from chromosomal DNA in a manner which is results in loss-of-function of the heterologous telomerase activator.

For example, the excision elements can be provided in the vector so as flank at least the coding sequence of a telomerase activator, though they may flank only a portion of the coding sequence such that the sequence resulting after excision does not encode a functional activator, or they may flank a sufficient portion of a transcriptional regulatory sequence for the telomerase activator such that resulting construct does not express the telomerase activator.

In preferred embodiments, the exicision elements are disposed in the vector such that, upon excision of the integrated form of the vector, no or substantially no portion (e.g., less than 50 nucleotides) of the vector DNA is left in the chromosomal DNA of the host cell.

In preferred embodiments, the transposition elements are viral transposition elements, e.g, retroviral or lentiviral transposition elements, such as may be provided where the vector is a replication-deficient virus.

In preferred embodiments, the excision elements comprise enzyme-assisted site-specific integration sequences. For instance, the excision elements may include recombinase target sites, e.g., recombinase target sites for Cre recombinase, Flp recombinase, Pin recombinase, lamda integrase, Gin recombinase or R recombinase. The excision elements may also be restriction enzyme sites.

In preferred embodiments, the vector is a retroviral vector which recombinase sites which are located in the LTRs such that excision of a proviral sequence occurs, e.g., the viral vector is completely, or nearly completely excised from the chromosomal DNA of the host cell.

The vector can include such other elements as: transcriptional regulatory sequences for directing transcription of the coding sequence for the telemorase activator cnucleic; a packaging signal for packaging the vector in an infectious viral particle;

Exemplary vectors of this type, e.g., readily excisable, are descibed in the appended examples as well as PCT publication WO 98/12339. On advantage that certain of these vectors have, e.g., those which can be substantially excised, can be realized for embodiments wherein the method is part of an *ex vivo* therapy. In such embodiments, the cells can be treated *ex vivo* with the constructs. Prior to implantation in a host, the cells are treated with an agent, such as a recombinase, which results in exicision of the vector from the genomic DNA of the host cell. Thus, the cells which are implanted are no longer genetically engineered. In such embodiments, it may be desirable to include one or more detectable genes (markers) on the vector in order to be able to identify cells which still retained the vector, e.g., by FACS sorting, affinity purification or other techniques.

The reversibility of telomerase activation can also be generated by use of an expression system which is inducible because of the presence of an inducible transcriptional regulatory sequence controlling the expression of the coding sequence of the EST or telomerase activator. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. Where the cells are to be transplanted into a patient, the inducible promoter is preferably one which is regulated by a small molecule or other factor which is not endogenous to the host animal.

Exemplary regulatable promoters include the tetracycline responsive promoters, such as described in, for example, Gossen et al. (1992) PNAS 89:5547-5551; and Pescini et al., (1994) Biochem. Biophys. Res. Comm. 202:1664-1667.

In another another embodiment, the subject method utilizes the multimerization technology first pioneered by Schreiber and Crabtree. This technique permits the regulation of expression of an endogenous or heterologous gene, in this case a coding sequence for EST or a telomerase activator, by use of chimeric transcription factors which are dependent on small molecules "dimerizers" to assemble transcriptionally active complexes. See, for example, PCT publications WO 9612796; WO 9505389; WO 9502684; WO 9418317; WO 9606097; and WO 9606110. Moreover, a number of techniques have been deveolped more recently which permit the recruitment of endogenous DNA binding and activation domains to the transcriptional regulatory sequences by use of artificial dimerization molecules. See, for example, PCT publication WO 9613613.

In other embodiments, the reversibility of telomerase activation can be accomplished by use of conditionally active (or conditionally inactivable) forms of EST or of the telomerase activators. For instance, temperature-sensitive mutants of telomerase or myc can be employed in the subject method. In embodiments wherein the cells are to be transplanted into an animal, the ts mutant can be inactive at body temperature (the non-permissive temperature) and active at a lower or higher cell culture temperature.

To illustrate, one strategy for producing temperature-sensitive EST or myc mutants, that does not require a search for a ts mutation in a gene of interest, is based on a portable, heat-inducible N-degron. The N-degron is an intracellular degradation signal whose essential determinant is a "destabilizing" N-terminal residue of a protein. A set of N-degrons containing different destabilizing residues is manifested as the N-end rule, which relates the in vivo half-life of a protein to the identity of its N-terminal residue. In eukaryotes, the N-degron consists of at least two determinants: a destabilizing N-terminal residue and a specific internal Lys residue (or residues) of a substrate. The Lys residue is the site of attachment of a multiubiquitin chain. Ubiquitin is a protein whose covalent conjugation to other proteins plays a role in a number of cellular processes, primarily through routes that involve protein degradation. For a description of exemplary heat-inducible N-degron modules which can be adapted for generating conditional mutants of EST, myc or other telomerase activators, see US Patents 5,705,387 and 5,538,862, and Dohmen et al. (1994) Science 263:1273-6.

In yet other embodiments, the multimerization technology referred to above can be used to generate small molecule inducible forms of EST or a telomerase activator. To illustrate, a first gene construct can be provided which encodes a fusion protein including a DNA binding domain (and optionally oligomerization domains) of myc and a ligand binding domain which binds to a small organic molecule, e.g., a domain which will bind to a dimerizing agent. A second gene construct is also provided, which construct encodes a fusion protein including an activation domain, e.g., a VP16 activation domain, and a ligand binding domain which will also bind the dimerizing agent when it is already bound to the first fusion protein. Expression of these two fusion proteins in a host cell, in the absence of the dimerizing agent, will not activate telomerase. Upon addition of the dimerizing agent, the fusion proteins associate, and activate transcription of genes which include myc responsive elements, which causes activation of telomerase activity.

In yet another embodiment, ectopic expression of EST2 or other telomerase activator can be by way of a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous telomearse activator gene. For instance, the gene activation construct can replace the endogenous promoter of an

EST2 gene with a heterologous promoter, e.g., one which causes consitutive expression of the EST2 gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. A vareity of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic gene upon recombination of the gene activation construct. The construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with, e.g., the native EST2 gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous EST2 gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regualtory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, J. Exp. Med., 169:13), the human β-actin promoter (Gunning et al. (1987) PNAS 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV

LTR) (Klessig et al. (1984) Mol. Cell Biol. 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) Nature 290:304-310; Templeton et al. (1984) Mol. Cell Biol., 4:817; and Sprague et al. (1983) J. Virol., 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) PNAS 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) Nature Genetics, 1:379-384).

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression.

In yet another embodiment, membrane permeable drugs (e.g., preferably small organic molecules) can be identified which activate the expression of an endogenous EST2 gene. In light of the availability of the genomic EST2 gene, it will be possible to produce reporter constructs in which a reporter gene is operably linked to the transcriptional regulatory seqence of the EST2 gene. When transfected into cells which possess the appropriate intracellular machinery for activation of the reporter construct through the EST2 regulatory sequence, the resulting cells can be used in a cell-based approach for identifying such compounds.

In embodiments wherein the cells are treated in culture, RNA encoding EST2, *myc* or another telomerase activator can be introduced directly into the cell, e.g., from RNA generated by *in vitro* transciption. In preferred embodiments, the RNA is preferably a modified polynucleotide which is resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases. Exemplary nucleic acid modifications which can be used to generate such RNA polynucleotides include phosphoramidate, phosphothioate and methylphosphonate analogs of nucleic acids (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775), or peptide nucleic acids (PNAs).

In still another embodiment of the subject method, the telomerase activator polypeptide can be contacted with a cell under conditions wherein the protein is taken up by the cell, e.g., internalized, without the need for recombinant expression in the cell. For instance, in the application of the subject method to skin, mucosa and the like, a variety of techniques have been developed for the transcytotic delivery of ectopically added proteins.

In an exemplary embodiment, the EST2 or *myc* protein is provided for transmucosal or transdermal delivery. For such administration, penetrants appropriate to the barrier to be permeated are used in the formulation with the polypeptide. Such penetrants are generally

known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the proteins of the invention are formulated into ointments, salves, gels, or creams as generally known in the art. For example, Chien et al. (1989) J. Pharm. Sci. 78:376-383 describes direct current iontophoretic transdermal delivery of peptide and protein drugs. Srinivasan et al., (1989) J. of Pharm. Sci. 78:370-375 describes the transdermal iontophoretic drug delivery : Mechanistic analysis and application to polypeptide delivery. See also USSN 4,940,456.

USSN 5,459,127 describes the use of cationic lipids for intracellular delivery of biologically active molecules.

USSN 5,190,762 describes methods of administering proteins to living skin cell.

In another embodiment, the polypeptide is provided as a chimeric polypeptide which includes a heterologous peptide sequence ("internalizing peptide") which drives the translocation of an extracellular form of a thereapeutic polypeptide sequence across a cell membrane in order to facilitate intracellular localization of the thereapeutic polypeptide. In this regard, the therapeutic polypeptide sequence is one which is active intracellularly, such as a tumor suppressor polypeptide, transcription factor or the like. The internalizing peptide, by itself, is capable of crossing a cellular membrane by, e.g., transcytosis, at a relatively high rate. The internalizing peptide is conjugated, e.g., as a fusion protein, to the telomerase activator polypeptide. The resulting chimeric polypeptide is transported into cells at a higher rate relative to the activator polypeptide alone to thereby provide an means for enhancing its introduction into cells to which it is applied, e.g., to enhance topical applications of the EST2 polypeptide.

In one embodiment, the internalizing peptide is derived from the drosopholia antepennepedia protein, or homologs thereof. The 60 amino acid long long homeodomain of the homeo-protein antepennepedia has been demonstrated to translocate through biological membranes and can facilitate the translocation of heterologous polypeptides to which it is couples. See for example Derossi et al. (1994) J Biol Chem 269:10444-10450; and Perez et al. (1992) J Cell Sci 102:717-722. Recently, it has been demonstrated that fragments as small as 16 amino acids long of this protein are sufficient to drive internalization. See Derossi et al. (1996) J Biol Chem 271:18188-18193. The present invention contemplates a chimeric protein comprising at least one EST2 or *myc* polypeptide sequence and at least a portion of the antepennepedia protein (or homolog thereof) sufficient to increase the transmembrane transport of the chimeric protein, relative to the EST2 or *myc* polypeptide, by a statistically significant amount.

Another example of an internalizing peptide is the HIV transactivator (TAT) protein. This protein appears to be divided into four domains (Kuppuswamy et al. (1989) Nucl. Acids Res. 17:3551-3561). Purified TAT protein is taken up by cells in tissue culture (Frankel and Pabo, (1989) Cell 55:1189-1193), and peptides, such as the fragment corresponding to residues 37 -62 of TAT, are rapidly taken up by cell *in vitro* (Green and Loewenstein, (1989) Cell 55:1179-1188). The highly basic region mediates internalization and targeting of the internalizing moiety to the nucleus (Ruben et al., (1989) J. Virol. 63:1-8). Peptides or analogs that include a sequence present in the highly basic region, such as CFITKALGISYGRKKRRQRRRPPQGS, are conjugated to EST2 or *myc* polypeptides to aid in internalization and targeting those proteins to the intracellular milleau.

Another exemplary transcellular polypeptide can be generated to include a sufficient portion of mastoparan (T. Higashijima et al., (1990) J. Biol. Chem. 265:14176) to increase the transmembrane transport of the chimeric protein.

While not wishing to be bound by any particular theory, it is noted that hydrophilic polypeptides may be also be physiologically transported across the membrane barriers by coupling or conjugating the polypeptide to a transportable peptide which is capable of crossing the membrane by receptor-mediated transcytosis. Suitable internalizing peptides of this type can be generated using all or a portion of, e.g., a histone, insulin, transferrin, basic albumin, prolactin and insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II) or other growth factors. For instance, it has been found that an insulin fragment, showing affinity for the insulin receptor on capillary cells, and being less effective than insulin in blood sugar reduction, is capable of transmembrane transport by receptor-mediated transcytosis and can therefor serve as an internalizing peptide for the subject transcellular polypeptides. Preferred growth factor-derived internalizing peptides include EGF (epidermal growth factor)-derived peptides, such as CMHIESLDSYTC and CMYIEALDKYAC; TGF- beta (transforming growth factor beta)-derived peptides; peptides derived from PDGF (platelet-derived growth factor) or PDGF-2; peptides derived from IGF-I (insulin-like growth factor) or IGF-II; and FGF (fibroblast growth factor)-derived peptides.

Another class of translocating/internalizing peptides exhibits pH-dependent membrane binding. For an internalizing peptide that assumes a helical conformation at an acidic pH, the internalizing peptide acquires the property of amphiphilicity, e.g., it has both hydrophobic and hydrophilic interfaces. More specifically, within a pH range of approximately 5.0-5.5, an internalizing peptide forms an alpha-helical, amphiphilic structure that facilitates insertion of the moiety into a target membrane. An alpha-helix-inducing acidic pH environment may be found, for example, in the low pH environment present within cellular endosomes. Such internalizing

peptides can be used to facilitate transport of telomerase activator polypeptides, taken up by an endocytic mechanism, from endosomal compartments to the cytoplasm.

A preferred pH-dependent membrane-binding internalizing peptide includes a high percentage of helix-forming residues, such as glutamate, methionine, alanine and leucine. In addition, a preferred internalizing peptide sequence includes ionizable residues having pKa's within the range of pH 5-7, so that a sufficient uncharged membrane-binding domain will be present within the peptide at pH 5 to allow insertion into the target cell membrane.

A particularly preferred pH-dependent membrane-binding internalizing peptide in this regard is aa1-aa2-aa3-EAALA(EALA)4-EALEALAA-amide, which represents a modification of the peptide sequence of Subbarao et al. (Biochemistry 26:2964, 1987). Within this peptide sequence, the first amino acid residue (aa1) is preferably a unique residue, such as cysteine or lysine, that facilitates chemical conjugation of the internalizing peptide to a targeting protein conjugate. Amino acid residues 2-3 may be selected to modulate the affinity of the internalizing peptide for different membranes. For instance, if both residues 2 and 3 are lys or arg, the internalizing peptide will have the capacity to bind to membranes or patches of lipids having a negative surface charge. If residues 2-3 are neutral amino acids, the internalizing peptide will insert into neutral membranes.

Yet other preferred internalizing peptides include peptides of apo-lipoprotein A-1 and B; peptide toxins, such as melittin, bombolittin, delta hemolysin and the pardaxins; antibiotic peptides, such as alamethicin; peptide hormones, such as calcitonin, corticotrophin releasing factor, beta endorphin, glucagon, parathyroid hormone, pancreatic polypeptide; and peptides corresponding to signal sequences of numerous secreted proteins. In addition, exemplary internalizing peptides may be modified through attachment of substituents that enhance the alpha-helical character of the internalizing peptide at acidic pH.

Yet another class of internalizing peptides suitable for use within the present invention include hydrophobic domains that are "hidden" at physiological pH, but are exposed in the low pH environment of the target cell endosome. Upon pH-induced unfolding and exposure of the hydrophobic domain, the moiety binds to lipid bilayers and effects translocation of the covalently linked polypeptide into the cell cytoplasm. Such internalizing peptides may be modeled after sequences identified in, e.g., Pseudomonas exotoxin A, clathrin, or Diphtheria toxin.

Pore-forming proteins or peptides may also serve as internalizing peptides herein. Pore-forming proteins or peptides may be obtained or derived from, for example, C9 complement protein, cytolytic T-cell molecules or NK-cell molecules. These moieties are capable of forming

ring-like structures in membranes, thereby allowing transport of attached polypeptide through the membrane and into the cell interior.

Mere membrane intercalation of an internalizing peptide may be sufficient for translocation of the polypeptide, e.g. EST2 or *myc*, across cell membranes. However, translocation may be improved by attaching to the internalizing peptide a substrate for intracellular enzymes (i.e., an "accessory peptide"). It is preferred that an accessory peptide be attached to a portion(s) of the internalizing peptide that protrudes through the cell membrane to the cytoplasmic face. The accessory peptide may be advantageously attached to one terminus of a translocating/internalizing moiety or anchoring peptide. An accessory moiety of the present invention may contain one or more amino acid residues. In one embodiment, an accessory moiety may provide a substrate for cellular phosphorylation (for instance, the accessory peptide may contain a tyrosine residue).

An exemplary accessory moiety in this regard would be a peptide substrate for N-myristoyl transferase, such as GNAAAARR (Eubanks et al., in: Peptides. Chemistry and Biology, Garland Marshall (ed.), ESCOM, Leiden, 1988, pp. 566-69) In this construct, an internalizing, peptide would be attached to the C-terminus of the accessory peptide, since the N-terminal glycine is critical for the accessory moiety's activity. This hybrid peptide, upon attachment to an EST2 or *myc* polypeptide at its C-terminus, is N-myristylated and further anchored to the target cell membrane, e.g., it serves to increase the local concentration of the polypeptide at the cell membrane.

To further illustrate use of an accessory peptide, a phosphorylatable accessory peptide is first covalently attached to the C-terminus of an internalizing peptide and then incorporated into a fusion protein with an EST2 or *myc* polypeptide. The peptide component of the fusion protein intercalates into the target cell plasma membrane and, as a result, the accessory peptide is translocated across the membrane and protrudes into the cytoplasm of the target cell. On the cytoplasmic side of the plasma membrane, the accessory peptide is phosphorylated by cellular kinases at neutral pH. Once phosphorylated, the accessory peptide acts to irreversibly anchor the fusion protein into the membrane. Localization to the cell surface membrane can enhance the translocation of the polypeptide into the cell cytoplasm.

Suitable accessory peptides include peptides that are kinase substrates, peptides that possess a single positive charge, and peptides that contain sequences which are glycosylated by membrane-bound glycotransferases. Accessory peptides that are glycosylated by membrane-bound glycotransferases may include the sequence x-NLT-x, where "x" may be another peptide, an amino acid, coupling agent or hydrophobic molecule, for example. When this hydrophobic

tripeptide is incubated with microsomal vesicles, it crosses vesicular membranes, is glycosylated on the luminal side, and is entrapped within the vesicles due to its hydrophilicity (C. Hirschberg et al., (1987) Ann. Rev. Biochem. 56:63-87). Accessory peptides that contain the sequence x-NLT-x thus will enhance target cell retention of corresponding polypeptide.

In another embodiment of this aspect of the invention, an accessory peptide can be used to enhance interaction of the telomerase activator polypeptide with the target cell. Exemplary accessory peptides in this regard include peptides derived from cell adhesion proteins containing the sequence "RGD", or peptides derived from laminin containing the sequence CDPGYIGSRC. Extracellular matrix glycoproteins, such as fibronectin and laminin, bind to cell surfaces through receptor-mediated processes. A tripeptide sequence, RGD, has been identified as necessary for binding to cell surface receptors. This sequence is present in fibronectin, vitronectin, C3bi of complement, von-Willebrand factor, EGF receptor, transforming growth factor beta , collagen type I, lambda receptor of E. coli, fibrinogen and Sindbis coat protein (E. Ruoslahti, Ann. Rev. Biochem. 57:375-413, 1988). Cell surface receptors that recognize RGD sequences have been grouped into a superfamily of related proteins designated "integrins". Binding of "RGD peptides" to cell surface integrins will promote cell-surface retention, and ultimately translocation, of the polypeptide.

As described above, the internalizing and accessory peptides can each, independently, be added to an EST2 or *myc* polypeptide by either chemical cross-linking or in the form of a fusion protein. In the instance of fusion proteins, unstructured polypeptide linkers can be included between each of the peptide moieties.

In general, the internalization peptide will be sufficient to also direct export of the polypeptide. However, where an accessory peptide is provided, such as an RGD sequence, it may be necessary to include a secretion signal sequence to direct export of the fusion protein from its host cell. In preferred embodiments, the secretion signal sequence is located at the extreme N-terminus, and is (optionally) flanked by a proteolytic site between the secretion signal and the rest of the fusion protein.

In an exemplary embodiment, an EST2 or *myc* polypeptide is engineered to include an integrin-binding RGD peptide/SV40 nuclear localization signal (see, for example Hart SL et al., 1994; J. Biol. Chem.,269:12468-12474), such as encoded by the nucleotide sequence provided in the Nde1-EcoR1 fragment: catatgggtggctgccgtggcgatatgttcggttgcggtgctcctccaaaaaagaagagaaag-gtagctggattc, which encodes the RGD/SV40 nucleotide sequence: MGGCRGDMFGCGAPP-KKKRKVAGF. In another embodiment, the protein can be engineered with the HIV-1 tat(1-72) polypeptide, e.g., as provided by the Nde1-EcoR1 fragment:catatggagccagtagatcctagactagagccc-

tggaagcatccaggaagtcagcctaaaactgcttgtaccaattgctattgtaaaaagtgttgctttcattgccaagtttgtttcataacaaaagcc cttggcatctcctatggcaggaagaagcggagacagcgacgaagacctcctcaaggcagtcagactcatcaagtttctctaagtaagcaag gattc, which encodes the HIV-1 tat(1-72) peptide sequence: MEPVDPRLEPWKHPGSQPKT-ACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQ. In still another embodiment, the fusion protein includes the HSV-1 VP22 polypeptide (Elliott G., O'Hare P (1997) Cell, 88:223-233) provided by the Nde1-EcoR1 fragment:

cat atg acc tct cgc cgc tcc gtg aag tcg ggt ccg cgg gag gtt ccg cgc gat gag tac gag gat ctg tac tac acc ccg tct tca ggt atg gcg agt ccc gat agt ccg cct gac acc tcc cgc cgt ggc gcc cta cag aca cgc tcg cgc cag agg ggc gag gtc cgt ttc gtc cag tac gac gag tcg gat tat gcc ctc tac ggg ggc tcg tca tcc gaa gac gac gaa cac ccg gag gtc ccc cgg acg cgg cgt ccc gtt tcc ggg gcg gtt ttg tcc ggc ccg ggg cct gcg cgg gcg cct ccg cca ccc gct ggg tcc gga ggg gcc gga cgc aca ccc acc acc gcc ccc cgg gcc ccc cga acc cag cgg gtg gcg act aag gcc ccc gcg gcc ccg gcg gcg gag acc acc cgc ggc agg aaa tcg gcc cag cca gaa tcc gcc gca ctc cca gac gcc ccc gcg tcg acg gcg cca acc cga tcc aag aca ccc gcg cag ggg ctg gcc aga aag ctg cac ttt agc acc gcc ccc cca aac ccc gac gcg cca tgg acc ccc cgg gtg gcc ggc ttt aac aag cgc gtc ttc tgc gcc gcg gtc ggg cgc ctg gcg gcc atg cat gcc cgg atg gcg gcg gtc cag ctc tgg gac atg tcg cgt ccg cgc aca gac gaa gac ctc aac gaa ctc ctt ggc atc acc acc atc cgc gtg acg gtc tgc gag ggc aaa aac ctg ctt cag cgc gcc aac gag ttg gtg aat cca gac gtg gtg cag gac gtc gac gcg gcc acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc acc gag cga cct cga gcc cca gcc cgc tcc gct tct cgc ccc aga cgg ccc gtc gag gaa ttc

which encodes the HSV-1 VP22 peptide having the sequence:

MTSRRSVKSGPREVPRDEYEDLYYTPSSGMASPDSPPDTSRRGALQTRSRQRGEVRFVQ
YDESDYALYGGSSSEDDEHPEVPRTRRPVSGAVLSGPGPARAPPPPAGSGGAGRTPTTA
PRAPRTGRVATKAPAAPAAETTRGRKSAQPESAALPDAPASTAPTRSKTPAQGLARKLH
FSTAPPNPDAPWTPRVAGFNKRVFCAAVGRLAAMHARMAAVQLWDMSRPRTDEDLN
ELLGITTIRVTVCEGKNLLQRANELVNPDVVQDVDAATATRGRSAASRPTERPRAPARS
ASRPRRPVE

In still another embodiment, the fusion protein includes the C-terminal domain of the VP22 protein from, e.g., the nucleotide sequence (Nde1-EcoR1 fragment):

cat atg gac gtc gac gcg gcc acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc acc gag cga cct cga gcc cca gcc cgc tcc gct tct cgc ccc aga cgg ccc gtc gag gaa ttc

which encodes the VP22 (C-terminal domain) peptide sequence:

MDVDAATATRGRSAASRPTERPRAPARSASRPRRPVE

In other embodiments, the subject method employs small, organic molecules, e.g., having a molecular weight of less than 5000 amu, more preferably less than 1000 amu, and even more preferably less than 500 amu. Moreover, such compounds are preferably membrane permeant, e.g., able to diffuse across the cell membrane into the host cell when added directly to culture cells or cells in whole blood.

In this regard, the art provides examples of assays for identifying agents which are capable of activating telomerase activity, e.g., see US Patents 5,837,453, 5,830,644, 5,804,380 and 5,686,245.

In yet another embodiment, to the extent it is relevant, the intracellular level of TRT or a telomerase activator (protein) can be upregulated by inhibiting its natural turnover rate. For example, inhibitors of ubiquitin-dependent or independent degradation of the protein can be used to cause ectopic expression of protein in the sense that the concentration of the protein in the cell can be artificially elevated. Assays for detecting inhibitors of ubiquitination, e.g., which can be readily adapted for detecting inhibitors of ubiquitination of *myc* or other telomerase activators, are described in the literature, as for example US Patents 5,744,343, 5,847,094, 5,847,076, 5,834,487, 5,817,494, 5,780,454 and 5,766,927. Likewise, to the extent that other post-translational modifications, such as phosphorylation, influence protein stability, the present invention contemplates the use of inhibitors of such modifications, including, as appropriate, kinase or phosphatase inhibitors.

In still other embodiments, cellular prolifeartive capacity can be incrased by contacting the cell with an agent, e.g. a small molecule, which relieves or otherwise inhibits a signal which antagonizes *myc*-induced activation of telomerase activity. For instance, agents can be used which disrupt protein-protein interactions involved in inhibition of *myc* activity by, e.g., *mad-max* heterodimers.

(B) Conjoint Applications

Another aspect of the invention provides a conjoint therapy wherein one or more other therapeutic agents are administered with the telomerase-activating therapeutic agent. Such conjoint treatment may be achieved by way of the simultaneous, sequential or separate dosing of the individual components of the treatment. For example, the telomerase-activating therapeutic agent can be administered conjointly with a growth factors and other mitogenic agents. Mitogenic agent, as used herein, refers to any compound or composition, including peptides, proteins, and glycoproteins, which is capable of stimulating proliferation of a target cell population. For example, the telomerase-activating therapeutic agent can be conjointly

administered with a T-cell mitogenic agent such as lectins, e.g., concanavalin A or phytohemagglutinin. Other exemplary mitogenic agents include insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and certain of the transforming growth factors (TGFs).

In one embodiment, the subject telomerase-activating therapeutic agent agent is co-administered with an agent that relieves "capping" inhibition of EST2 rescue. We have noticed that EST2 wiill neither extend telomere length nor lifespan in late-passage HMEC cells, and certain other cell lines such as fibroblasts. While not wishing to be bound by any particular theory, this inability to extend telomeres in such cells may be the result of reaction kinetics –e.g., telomere binding proteins such as TRF (TTAGGG repeat binding factor) become abundant relevant to the telomeric sequences. The increased loading of telomeres with such proteins inhibits elongation induced by ectopic EST2. Such relative overabundance of proteins to telomers may be the result of, for example, reduction in the number of telomeric sequences relative to a constant concentration of associated proteins, increased expression (or stability) of the associated proteins, or a combination thereof. To alleviate such kinetic inhibition of EST2 activity, the cells can be treated with an oligonucleotide which competes (e.g., as a decoy) with the telomeres for binding of the telomere binding proteins. See, for example, Wright et al. (1996) EMBO J 15: 1734. In other embodiments, a dominant negative mutant of a telomere binding protein can be introduced into the cell in order to inhibit the formation of inhibitory protein complexes with the telomeric sequences. See, for example, Bianchi et al. (1997) EMBO J 16:1785-94; Broccoli et al. (1997) Hum Mol Genet 6: 69-76; Smith et al. (1997) Trends Genet 13:21-26; Zhong et al, (1992) Mol. Cell. Biol. 12:4834-4843; Chong et al. (1995) Science 270:1663-166). In still other embodiments, the agent can be an inhibitor of expression of a telomere binding proteins, such as antisense or a small molecule inhibitor of transcription of the gene. In yet other embodiments, such agents, particularly small molecules, can be identified by their ability to directly inhibit the formation of telomeric complexes including telomere binding proteins.

(C) Exemplary Uses of the Subject Method

The present method can be used to increase the proliferative capacity of cells *in vivo*, *in vitro* and as part of an ex vivo protocol. While the method of the invention is applicable to any normal cell type, the method is preferably practiced using normal cells that express a low level of telomerase activity. For purposes of the present invention, the term "normal" refers to cells other than tumor cells, cancer calls, or transformed cells. An exemplary cell is an embryonic

stem cells, such as disclosed in Thomson et al. (1998) Science 282:1145 and Shamblott et al. (1998) PNAS 95:13726. Especially preferred cells for use in the present method include embryonic, fetal, neonatal, and adult stem cells of any organ, and adult pluripotent hematopoietic stem cells.

In one embodiment, the cells are stem and/or progenitor cells. These include hematopoietic stem cells, e.g., which are derived from bone marrow, mobilized peripheral blood cells, or cord blood. In other embodiments, the cells are progenitor cells for pancreatic or hepatic tissue, or other tissue deriving from the primative gut. In still other embodiments, the stem is a neuronal stem cell, such as neural crest which can be used to form neurons or smooth muscle cells.

In other embodiments, the cells are not stem or progenitor cells, e.g., they are committed cells, such as pancreatic β cells, smooth muscle cells (or other myocytic cells), fibroblasts, lymphocytic cells, e.g., B or T cells, osteocytes or chondrocytes, to name but a few.

While the subject method can be used either *in vivo* or *in vitro*, the invention has particular application to the cultivation of cells *ex vivo*, and provides especially important benefits to therapeutic methods in which cells are cultured *ex vivo* and then reintroduced to a host. For example, the subject method can be used to extend the proliferative capacity of cells which are harvested, or otherwise isolated in culture, which are to be transplanted to a patient.

Such protocols can find use in bone marrow transplants wherein bone marrow, or isolated hematopoietic progenitor cells are treated according to the present invention, with the activation of telomerase and inactivation of Rb being reverted to the wild-type phenotype before, or shortly after, transplantation.

The subject method can also be used to extend T cell life in HIV and Down's patients.

It also has application in protocols for the formation of artificial tissues such as prosthetic devices, e.g., deriving from stem or committed cells. Exemplary tissues include pancreatic, hepatic, neural, myocytic, cartilaginous and osseous tissue.

To illustrate, the subject method can be used to enhance the lifespan of a hematopoietic cells and hematopoietic stem/progenitor cells. The term "hematopoietic cells" herein refers to fully differentiated myeloid cells such as erythrocytes or red blood cells, megakaryocytes, monocytes, granulocytes, and eosinophils, as well as fully differentiated lymphoid cells such as B lymphocytes and T lymphocytes. Thus, a hematopoietic stem/progenitor cell includes the various hematopoietic precursor cells from which these differentiated cells develop, such as BFU-E (burst-forming units-erythroid), CFU-E (colony forming unit-erythroid), CFU-Meg

(colony forming unit-megakaryocyte), CFU-GM (colony forming unit-granulocyte-monocyte), CFU-Eo (colony forming unit-eosinophil), and CFU-GEMM (colony forming unit-granulocyte-erythrocyte-megakaryocyte-monocyte).

In another embodiment, the subject method can be use to extend the lifespan of a pancreatic cells and pancreatic stem/progenitor cells. The term "pancreatic progenitor cell" refers to a cell which can differentiate into a cell of pancreatic lineage, e.g. a cell which can produce a hormone or enzyme normally produced by a pancreatic cell. For instance, a pancreatic progenitor cell may be caused to differentiate, at least partially, into α, β, δ, or ϕ islet cell, or a cell of exocrine fate. The pancreatic progenitor cells of the invention can also be cultured prior to administration to a subject under conditions which promote cell proliferation and differentiation. These conditions include culturing the cells to allow proliferation and confluence *in vitro* at which time the cells can be made to form pseudo islet-like aggregates or clusters and secrete insulin, glucagon, and somatostatin.

The endocrine portion of the pancreas is composed of the islets of Langerhans. The islets of Langerhans appear as rounded clusters of cells embedded within the exocrine pancreas. Four different types of cells- α, β, δ, and ϕ-have been identified in the islets. The α cells constitute about 20% of the cells found in pancreatic islets and produce the hormone glucagon. Glucagon acts on several tissues to make energy available in the intervals between feeding. In the liver, glucagon causes breakdown of glycogen and promotes gluconeogenesis from amino acid precursors. The δ cells produce somatostatin which acts in the pancreas to inhibit glucagon release and to decrease pancreatic exocrine secretion. The hormone pancreatic polypeptide is produced in the ϕ cells. This hormone inhibits pancreatic exocrine secretion of bicarbonate and enzymes, causes relaxation of the gallbladder, and decreases bile secretion. The most abundant cell in the islets, constituting 60-80% of the cells, is the β cell, which produces insulin. Insulin is known to cause the storage of excess nutrients arising during and shortly after feeding. The major target organs for insulin are the liver, muscle, and fat-organs specialized for storage of energy.

In an exemplary embodiment, the subject telomerase-activating therapeutic agents can be used to extend the lifespan of implanted pancreatic tissue, e.g., implanted β-islet cells. Recently, tissue-engineering approaches to treatment have focused on transplanting pancreatic islets, usually encapsulated in a membrane to avoid immune rejection. Many methods for encapsulating cells are known in the art. For example, a source of β islet cells producing insulin is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the β islet cells (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) Expt. Neurobiol. 110:39-44; Jaeger et al. (1990) Prog. Brain

Res. 82:41-46; and Aebischer et al. (1991) J. Biomech. Eng. 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the β islet cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) Trans. Am. Artif. Intern. Organs 35:791-799; Sefton et al. (1987) Biotehnol. Bioeng. 29:1135-1143; and Aebischer et al. (1991) Biomaterials 12:50-55).

In any of the above-embodiments, the pancreatic cells can be treated by the subject method ex vivo, and/or treated by the subject method by subsequent delivery of an therapeutic to an animal in which the device is implanted. Such cells can be used for treatment of diabetes because they have the ability to differentiate into cells of pancreatic lineage, e.g., β islet cells. The pancreatic cells of the invention can be cultured *in vitro* under conditions which can further induce these cells to differentiate into mature pancreatic cells, or they can undergo differentiation in vivo once introduced into a subject.

Moreover, in addition to providing a source of implantable cells, either in the form of the progenitor cell population of the differentiated progeny thereof, the subject method can be used to extend the life of normal pancreatic cells used to produce cultures for the production and purification of secreted factors. For instance, cultured cells can be provided as a source of insulin. Likewise, exocrine cultures can be provided as a source for pancreatin.

In still another embodiment, the subject method can be used to extend the life span of hepatic cells and hepatic stem cells. The term "hepatic progenitor cell" as used herein refers to a cell which can differentiate in a cell of hepatic lineage, such a liver parenchymal cell, e.g., a hepatocyte. Hepatocytes are some of the most versatile cells in the body. Hepatocytes have both endocrine and exocrine functions, and synthesize and accumulate certain substance, detoxify others, and secrete others to perform enzymatic, transport, or hormonal activities. The main activities of liver cells include bile secretion, regulation of carbohydrate, lipid, and protein metabolism, storage of substances important in metabolism, degradation and secretion of hormones, and transformation and excretion of drugs and toxins. The subject method can be used to facilitate the long term culture of hepatic cells and hepatic progenitor cells either in vitro or subsequent to implantation.

In still another embodiment, the subject method can be used to enhance the life of "feeder" cell layers for cell co-cultures.

In another embodiment, the subject method can be used to enhance large-scale cloning, e.g., of non-human animals, by enhancing the presence of actively dividing fetal fibroblasts for nuclear transfer.

Prior research in nuclear transplantation has shown that the cell cycle stage of the donor cell affects the extent of development of the embryo after nuclear transfer. When the donor cell is fused to the recipient oocyte, which is arrested in the second metaphase in meiosis, the nuclear envelope breaks down and the chromosomes condense until the oocyte is activated. This condensation phase has been shown to cause chromosomal defects in donor cells that are undergoing DNA synthesis. Donor cells in the G_1 phase of the cell cycle (before DNA synthesis), however, condense normally and support a high rate of early development.

Our rationale in selecting an optimal donor cell for nuclear transplantation was that the cell should not have ceased dividing (which is the case in G_0) but be actively dividing, as an indication of a relatively undifferentiated state and for compatibility with the rapid cell divisions that occur during early embryo development. The cells should also be in G_1, either by artificially arresting the cell cycle or by choosing a cell type that has an inherently long G1 phase.

The subject methods are also applicable to general cell culture techniques. For example, the method can be used to increase the replicative capacity of hybrids between immortal and mortal human cells, such as hybrids between human B-lymphocytes and myeloma cells, e.g., to increase the replicative capacity of antibody producing human hybridomas.

More generally, the subject method can be used to increase the replicative capacity of cells in culture which have been engineered to produce recombinant proteins. Indeed, the subject method can permit the use of "normal" cells as the recombinant cell, so that problems which may occur with the use of immortal cells (such as differences in post-translation modifications) can be avoided, particularly for producing secreted proteins.

In another aspect, the present invention provides pharmaceutical preparations and methods for controlling the proliferation of epithelially-derived tissue utilizing, as an active ingredient, a telomerase-activating therapeutic agent. The invention also relates to methods of controlling proliferation of epithelial-derived tissue by use of the pharmaceutical preparations of the invention. To illustrate, a telomerase-activating therapeutic agent of the present invention may be used as part of regimens in the treatment of disorders of, or surgical or cosmetic repair of, such epithelial tissues as skin and skin organs; corneal, lens and other ocular tissue; mucosal membranes; and periodontal epithelium. The methods and compositions disclosed herein provide for the treatment or prevention of a variety of damaged epithelial and mucosal tissues. For instance, the subject method can be used to control wound healing processes, as for example may be desirable in connection with any surgery involving epithelial tissue, such as from dermatological or periodontal surgeries. Exemplary surgical repair for which use of a telomerase-activating therapeutic agent is a candidate treatment include severe burn and skin

regeneration, skin grafts, pressure sores, dermal ulcers, fissures, post surgery scar reduction, and ulcerative colitis.

In another aspect of the present invention, telomerase-activating therapeutic agents can be used to effect the growth of hair, as for example in the treatment of alopecia whereby hair growth is potentiated or otherwise extended.

Still another aspect of the present invention provides a method of extending the lifetime of epithelial tissue in tissue culture.

The terms "epithelia", "epithelial" and "epithelium" refer to the cellular covering of internal and external body surfaces (cutaneous, mucous and serous), including the glands and other structures derived therefrom, e.g., corneal, esophegeal, epidermal, and hair follicle epithelial cells. Other exemplary epithlelial tissue includes: olfactory epithelium, which is the pseudostratified epithelium lining the olfactory region of the nasal cavity, and containing the receptors for the sense of smell; glandular epithelium, which refers to epithelium composed of secreting cells; squamous epithelium, which refers to epithelium composed of flattened plate-like cells. The term epithelium can also refer to transitional epithelium, which that characteristically found lining hollow organs that are subject to great mechanical change due to contraction and distention, e.g. tissue which represents a transition between stratified squamous and columnar epithelium.

The term "epithelialization" refers to healing by the growth of epithelial tissue over a denuded surface.

The term "skin" refers to the outer protective covering of the body, consisting of the corium and the epidermis, and is understood to include sweat and sebaceous glands, as well as hair follicle structures. Throughout the present application, the adjective "cutaneous" may be used, and should be understood to refer generally to attributes of the skin, as appropriate to the context in which they are used.

The term "epidermis" refers to the outermost and nonvascular layer of the skin, derived from the embryonic ectoderm, varying in thickness from 0.07-1.4 mm. On the palmar and plantar surfaces it comprises, from within outward, five layers: basal layer composed of columnar cells arranged perpendicularly; prickle-cell or spinous layer composed of flattened polyhedral cells with short processes or spines; granular layer composed of flattened granular cells; clear layer composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and horny layer composed of flattened, cornified non-nucleated cells. In the epidermis of the general body surface, the clear layer is usually absent.

The "corium" or "dermis" refers to the layer of the skin deep to the epidermis, consisting of a dense bed of vascular connective tissue, and containing the nerves and terminal organs of sensation. The hair roots, and sebaceous and sweat glands are structures of the epidermis which are deeply embedded in the dermis.

The term "hair" refers to a threadlike structure, especially the specialized epidermal structure composed of keratin and developing from a papilla sunk in the corium, produced only by mammals and characteristic of that group of animals. Also, the aggregate of such hairs. A "hair follicle" refers to one of the tubular-invaginations of the epidermis enclosing the hairs, and from which the hairs grow; and "hair follicle epithelial cells" refers to epithelial cells which surround the dermal papilla in the hair follicle, e.g., stem cells, outer root sheath cells, matrix cells, and inner root sheath cells. Such cells may be normal non-malignant cells, or transformed/immortalized cells.

"Excisional wounds" include tears, abrasions, cuts, punctures or lacerations in the epithelial layer of the skin and may extend into the dermal layer and even into subcutaneous fat and beyond. Excisional wounds can result from surgical procedures or from accidental penetration of the skin.

"Burn wounds" refer to cases where large surface areas of skin have been removed or lost from an individual due to heat and/or chemical agents.

"Dermal skin ulcers" refer to lesions on the skin caused by superficial loss of tissue, usually with inflammation. Dermal skin ulcers which can be treated by the method of the present invention include decubitus ulcers, diabetic ulcers, venous stasis ulcers and arterial ulcers. Decubitus wounds refer to chronic ulcers that result from pressure applied to areas of the skin for extended periods of time. Wounds of this type are often called bedsores or pressure sores. Venous stasis ulcers result from the stagnation of blood or other fluids from defective veins. Arterial ulcers refer to necrotic skin in the area around arteries having poor blood flow.

"Dental tissue" refers to tissue in the mouth which is similar to epithelial tissue, for example gum tissue. The method of the present invention is useful for treating periodontal disease.

"Internal epithelial tissue" refers to tissue inside the body which has characteristics similar to the epidermal layer in the skin. Examples include the lining of the intestine. The method of the present invention is useful for promoting the healing of certain internal wounds, for example wounds resulting from surgery.

A "wound to eye tissue" refers to severe dry eye syndrome, corneal ulcers and abrasions and ophthalmic surgical wounds.

The subject method has wide applicability to the treatment or prophylaxis of disorders afflicting epithelial tissue, as well as in cosmetic uses. In general, the method can be characterized as including a step of contacting a cell, in vitro or in vivo, with an amount of an telomerase-activating therapeutic agent agent sufficient to alter the life span of the treated epithelial tissue. For in vivo use, the mode of administration and dosage regimens will vary depending on the epithelial tissue(s) which is to be treated. For example, topical formulations will be preferred where the treated tissue is epidermal tissue, such as dermal or mucosal tissues.

A method which "promotes the healing of a wound" results in the wound healing more quickly as a result of the treatment than a similar wound heals in the absence of the treatment. "Promotion of wound healing" can also mean that the method causes the extends the proliferative and growth phase of, *inter alia,* keratinocytes, or that the wound heals with less scarring, less wound contraction, less collagen deposition and more superficial surface area. In certain instances, "promotion of wound healing" can also mean that certain methods of wound healing have improved success rates, (e.g. the take rates of skin grafts,) when used together with the method of the present invention.

Complications are a constant risk with wounds that have not fully healed and remain open. Although most wounds heal quickly without treatment, some types of wounds resist healing. Wounds which cover large surface areas also remain open for extended periods of time. In one embodiment of the present invention, the subject method can be used to enhance and/or otherwise accelerate the healing of wounds involving epithelial tissues, such as resulting from surgery, burns, inflammation or irritation. The telomerase-activating therapeutic agent agents of the present invention can also be applied prophylactically, such as in the form of a cosmetic preparation, to enhance tissue regeneration processes, e.g., of the skin, hair and/or fingernails.

Full and partial thickness burns are an example of a wound type which often covers large surface areas and therefore requires prolonged periods of time to heal. As a result, life-threatening complications such as infection and loss of bodily fluids often arise. In addition, healing in burns is often disorderly, resulting in scarring and disfigurement. In some cases wound contraction due to excessive collagen deposition results in reduced mobility of muscles in the vicinity of the wound. The compositions and method of the present invention can be used to enhance the healing of burns and to promote healing processes that result in more desirable cosmetic outcomes and less wound contraction and scarring.

Severe burns which cover large areas are often treated by skin autografts taken from undamaged areas of the patient's body. The subject method can also be used in conjunction with skin grafts to impove the grafts performance and life span in culture, as well as improve the "take" rates of the graft by accelerating growth of both the grafted skin and the patient's skin that is proximal to the graft.

Dermal ulcers are yet another example of wounds that are amenable to treatment by the subject method, e.g., to cause healing of the ulcer and/or to prevent the ulcer from becoming a chronic wound. For example, one in seven individuals with diabetes develop dermal ulcers on their extremities, which are susceptible to infection. Individuals with infected diabetic ulcers often require hospitalization, intensive services, expensive antibiotics, and, in some cases, amputation. Dermal ulcers, such as those resulting from venous disease (venous stasis ulcers), excessive pressure (decubitus ulcers) and arterial ulcers also resist healing. The prior art treatments are generally limited to keeping the wound protected, free of infection and, in some cases, to restore blood flow by vascular surgery. According to the present method, the afflicted area of skin can be treated by a therapy which includes a telomerase-activating therapeutic agent agent which promotes epithelization of the wound, e.g., accelerates the rate of the healing of the skin ulcers.

In another exemplary embodiment, the subject method is provided for treating or preventing gastrointestinal diseases. Briefly, a wide variety of diseases are associated with disruption of the gastrointestinal epithelium or villi, including chemotherapy- and radiation-therapy-induced enteritis (i.e. gut toxicity) and mucositis, peptic ulcer disease, gastroenteritis and colitis, villus atrophic disorders, and the like. For example, chemotherapeutic agents and radiation therapy used in bone marrow transplantation and cancer therapy affect rapidly proliferating cells in both the hematopoietic tissues and small intestine, leading to severe and often dose-limiting toxicities. Damage to the small intestine mucosal barrier results in serious complications of bleeding and sepsis. The subject method can be used to promote proliferation of gastrointenstinal epithelium and thereby increase the tolerated doses for radiation and chemotherapy agents. Effective treatment of gastrointestinal diseases may be determined by several criteria, including an enteritis score, other tests well known in the art.

With age, the epidermis thins and the skin appendages atrophy. Hair becomes sparse and sebaceous secretions decrease, with consequent susceptibility to dryness, chapping, and fissuring. The dermis diminishes with loss of elastic and collagen fibers. Moreover, keratinocyte proliferation (which is indicative of skin thickness and skin proliferative capacity) decreases with age. An increase, or prolinged rate of keratinocyte proliferation is believed to conteract skin aging, i.e., wrinkles, thickness, elasticity and repair. According to the present invention, a

telomerase-activating therapeutic agent can be used either therapeutically or cosmetically to counteract, at least for a time, the effects of aging on skin.

The subject method can also be used in treatment of a wound to eye tissue. Generally, damage to corneal tissue, whether by disease, surgery or injury, may affect epithelial and/or endothelial cells, depending on the nature of the wound. Corneal epithelial cells are the non-keratinized epithelial cells lining the external surface of the cornea and provide a protective barrier against the external environment. Corneal wound healing has been of concern to both clinicians and researchers. Opthomologists are frequently confronted with corneal dystrophies and problematic injuries that result in persistent and recurrent epithelial erosion, often leading to permanent endothelial loss. The use of telomerase-activating therapeutic agents can be used in these instances to promote epithelialization of the affected corneal tissue. To further illustrate, specific disorders typically associated with epithelial cell damage in the eye, and for which the subject method can provide beneficial treatment, include persistent corneal epithelial defects, recurrent erosions, neurotrophic corneal ulcers, keratoconjunctivitis sicca, microbial corneal ulcers, viral cornea ulcers, and the like. Moreover, superficial wounds such as scrapes, surface erosion, inflammation, etc. can cause lose of epithelial cells. According to the present invention, the corneal epithelium is contacted with an amount of a telomerase-activating therapeutic agent effective to enhance proliferation of the corneal epithelial cells to appropriately heal the wound.

The maintenance of tissues and organs *ex vivo* is also highly desirable. Tissue replacement therapy is well established in the treatment of human disease. For example, more than 40,000 corneal transplants were performed in the United States in 1996. Human epidermal cells can be grown in vitro and used to populate burn sites and chronic skin ulcers and other dermal wounds. The subject method can be used to enhance the life span of epithelial tissue *in vitro*, as well as to enhance the grafting of the cultured epithelial tissue to an animal host

The present method can be used for improving the "take rate" of a skin graft. Grafts of epidermal tissue can, if the take rate of the graft is to long, blister and shear, decreasing the likelihood that the autograft will "take", i.e. adhere to the wound and form a basement membrane with the underlying granulation tissue. Take rates can be increased by the subject method by enhancing the proliferation of the keratinocytes. The method of increasing take rates comprises contacting the skin autograft with an effective wound healing amount of a telomerase-activating therapeutic agent described in the method of promoting wound healing and in the method of promoting the growth and proliferation of keratinocytes, as described above.

Skin equivalents have many uses not only as a replacement for human or animal skin for skin grafting, but also as test skin for determining the effects of pharmaceutical substances and

cosmetics on skin. A major difficulty in pharmacological, chemical and cosmetic testing is the difficulties in determining the efficacy and safety of the products on skin. One advantage of the skin equivalents of the invention is their use as an indicator of the effects produced by such substances through in vitro testing on test skin.

Thus, in one embodiment of the subject method can be used as part of a protocol for skin grafting of, e.g., denuded areas, granulating wounds and burns. The use of telomerase-activating therapeutic agents can enhance such grafting techniques as split thickness autografts and epidermal autografts (cultured autogenic keratinocytes) and epidermal allografts (cultured allogenic keratinocytes). In the instance of the allograft, the use of the subject method to enhance the formation of skin equivalents in culture helps to provide/maintain a ready supply of such grafts (e.g., in tissue banks) so that the patients might be covered in a single procedure with a material which allows permanent healing to occur.

In this regard, the present invention also concerns composite living skin equivalents comprising an epidermal layer of cultured keratinocyte cells which have been expanded in the presence of a telomerase-activating therapeutic agent. The subject method can be used as part of a process for the preparation of composite living skin equivalents. In an illustrative embodiment, such a method comprises obtaining a skin sample, treating the skin sample enzymically to separate the epidermis from the dermis, treating the epidermis enzymically to release the keratinocyte cells, culturing, in the presence of a telomerase-activating therapeutic agent, the epidermal keratinocytes until confluence, in parallel, or separately, treating the dermis enzymatically to release the fibroblast cells, culturing the fibroblasts cells until sub-confluence, inoculating a porous, cross-linked collagen sponge membrane with the cultured fibroblast cells, incubating the inoculated collagen sponge on its surface to allow the growth of the fibroblast cells throughout the collagen sponge, and then inoculating it with cultured keratinocyte cells, and further incubating the composite skin equivalent complex in the presence of a telomerase-activating therapeutic agent to enhance the life span of the cells.

In other embodiments, skin sheets containing both epithelial and mesenchymal layers can be isolated in culture and expanded with culture media supplemented with a telomerase-activating therapeutic agent.

Any skin sample amenable to cell culture techniques can be used in accordance with the present invention. The skin samples may be autogenic or allogenic.

In another aspect of the invention, the subject method can be used in conjunction with various periodontal procedures in which control of epithelial cell proliferation in and around periodontal tissue is desired. In one embodiment, proliferative forms of the hedgehog and ptc

therapeutics can be used to enhance reepithelialization around natural and prosthetic teeth, e.g., to promote formation of gum tissue.

In yet another aspect, the subject method can be used to help control guided tissue regeneration, such as when used in conjunction with bioresorptable materials. For example, incorporation of periodontal implants, such as prosthetic teeth, can be facilitated by the instant method. Reattachment of a tooth involves both formation of connective tissue fibers and re-epithelization of the tooth pocket. The subject method treatment can be used to enhance tissue reattachment by controlling the mitotic capacity of basal epithelial cells in the wound healing process.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Telomere maintenance has been proposed as an essential prerequisite to human tumor development. The telomerase enzyme is itself a specific marker for tumor cells, but the genetic alterations that activate the enzyme during neoplastic tranformation have remained a mystery. Amplification of the *myc* oncogene is prevalent in a broad spectrum of human tumors. Here, we show that *myc* induces telomerase both in normal human mammary epithelial cells (HMEC) and in normal human diploid fibroblasts. *Myc* increases expression of hEST2 (hEST/TP2), the catalytic subunit of telomerase. Since hEST2 limits enzyme activity in normal cells, *myc* may control telomerase solely by regulating hEST2 levels. Activation of telomerase through hEST2 is sufficient to increase average telomere length and extend lifespan in normal human mammary epithelial cells. Since *myc* can also extend the lifespan of these cells, activation of telomerase may be one mechanism by which *myc* contributes to tumor formation.

Telomerase activity is largely absent from somatic cells in vivo and from normal human cells in culture[1]. As these cells proliferate, telomeric repeats are progressively lost due to the incomplete replication of chromosome ends during each division cycle [2-5]. Telomere shortening has been proposed as the mitotic clock that marks the progress of a cell toward the end of its replicative life-span. According to this model, erosion of chromosome ends triggers cellular senescence [6]. Bypass of senescence through negation of tumor suppressor pathways

(e.g. p53 and Rb/p16) allows continued proliferation and further loss of telomeric sequences [5, 7]. Indefinite proliferation in the absence of telomere maintenance would result in chromosomal destabilization due to complete loss of telomeres[8]. Since this is probably incompatible with survival, cells with an indeterminate life span must adopt strategies for telomere conservation [1, 9, 10].

Stabilization of telomeric repeats has been proposed as a prerequisite for tumorigenesis [11]. Circumstantial support for this notion comes from the observation that telomerase is activated in a high percentage of late-stage human tumors [1, 11, 12]. The possibility that telomere maintenance might be an essential component of the tumorigenic phenotype led us to survey known oncogenes for the ability to activate the telomerase enzyme.

Normal human mammary epithelial cells lack telomerase, whereas immortal HMEC-derivatives and breast tumor cell lines are almost universally telomerase-positive [13-15]. Introduction into HMEC of HPV-16 E6 protein stimulates telomerase activity, suggesting that, in these cells, a single genetic event can potentiate the enzyme [16, 17] (Fig. 3). HMEC were therefore used for the oncogene survey. Ectopic expression of mdm-2 failed to induce telomerase, consistent with the observation that activation of telomerase by E6 is separable from the ability of E6 to promote the degradation of p53[16] (data not shown). Several other cellular and viral oncogenes, including E7, activated ras (V12) and all cdc25 isoforms, also failed to induce telomerase (Fig 3, data not shown). However, introduction of a c-*myc* expression cassette resulted in the appearance of telomerase activity in HMEC (Fig. 3). The enzyme was detectable within one passage after transduction of HMEC with a retrovirus that directs *myc* expression. Following drug selection of infected cells, the *myc*-expressing population contained levels of telomerase activity that approximated those seen in a random sample of breast carcinoma cell lines (Fig. 3; e. g. T47D).

Introduction of E6 into normal human diploid fibroblasts fails to activate telomerase[16, 17] (Fig. 4). Similar results were observed following transfer of either activated ras or a dominant-negative p53 allele (data not shown). However, telomerase was induced by transduction of either IMR-90 (Fig. 4) or WI-38 cells (not shown) with a retrovirus that directs *myc* expression. As with HMEC, activity was apparent immediately after infection, and following selection of the *myc*-expressing population, telomerase reached levels comparable to those seen in a telomerase-positive fibrosarcoma cell line, HT1080 (Fig. 4).

A recent report suggests that E6 can activate the *myc* promoter [18]. This prompted us to ask whether E6 might regulate telomerase through an effect on *myc* expression. In HMEC, expression of E6 resulted in induction of *myc* to levels approaching those achieved upon

transduction of HMEC with a retrovirus that directs *myc* expression (Fig. 5A). Surprisingly, E6-induced alterations in *myc* protein did not reflect changes in the abundance of *myc* mRNA (Fig. 5B), suggesting that control of *myc* expression by E6 must occur at the post-transcriptional level. In contrast, *myc* levels remained unaltered following expression of E6 in IMR-90 cells wherein E6 is incapable of activating telomerase (Fig. 5A). This result is consistent with a model in which E6 regulates telomerase in HMEC by altering the abundance of *myc*.

The presence of the mRNA encoding hEST2, the catalytic subunit of telomerase, strictly correlates with telomerase activity. The mRNA for hEST2 is undetectable in normal tissue and in normal cell lines, whereas hEST2 is present in immortal and tumor-derived cell lines [19-21]. Moreover, hEST2 expression and telomerase are concomitantly suppressed when cells are induced to differentiate [20]. As expected, hEST2 mRNA was absent from normal HMEC. However, hEST2 could be detected in HMEC cells following transduction with a *myc* retrovirus (Fig. 6A). To determine whether increased expression of hEST2 was sufficient to account for activation of telomerase by *myc*, we infected HMEC and IMR-90 with a retrovirus that directs expression of hEST2. Delivery of hEST2 resulted in a clear induction of telomerase in both cell types (Fig. 6B). Considered together, our results indicate that *myc* regulates telomerase by controlling the expression of a limiting telomerase subunit. *Myc* is a transcription factor that can enhance the expression of responsive genes. Thus, *myc* could increase hEST2 expression by directly stimulating the hEST2 promoter. Alternatively, changes in hEST2 expression could arise as a secondary consequence of the ability of *myc* to regulate other genes.

Telomere length is regulated at two distinct levels. First, preservation of telomeric repeats requires either the telomerase enzyme or the activation of an alternative pathway for telomere maintenance [1, 9, 10, 14, 22]. Second, telomere length can be controlled by telomere binding proteins [23]. To determine whether activation of telomerase in HMEC cells is sufficient to stabilize telomere length, we followed telomeric restriction fragment (TRF) size as HMEC were passaged either in the presence or absence of telomerase activity. In normal HMEC, telomere length diminished slightly as cells underwent multiple rounds of division (Fig. 6C). Activation of telomerase by expression of hEST2 not only prevented telomere shrinkage but also increased average TRF length over that observed in early-passage cells (Fig. 6C).

Telomere length has been proposed as the counting mechanism that determines the replicative lifespan of a cell. Early-passage, normal HMEC which recieved either hEST2 or *myc* expression cassettes display extended lifespan as compared to vector-transduced cells (Fig. 6D). This supports the notion that telomere length is one of the criteria used by a cell to calculate its proliferative capacity.

Here we show that ectopic expression of *myc* can induce telomerase both in normal epithelial cells and in normal fibroblasts and can extend the replicative lifespan of HMEC. The *myc* oncogene is activated by gene amplification and possibly by mutation in a wide variety of different tumor types [24, 25]. Since *myc* can elevate telomerase to a level approximating that observed in tumor cell lines, increased *myc* activity could account for the presence of telomerase in many late-stage tumors. In this regard, a study of 100 neuroblastomas revealed that ~20% (16/100) had exceptionally high telomerase activity. Of these, 11 showed amplification of the N-*myc* locus [26]. Thus, in this case, telomerase levels correlated well with *myc* activation. Although the *myc* oncogene may induce telomerase in significant proportion of tumors, the enzyme may also be regulated by other pathways [27].

Promotion of cell proliferation and oncogenic transformation by *myc* probably requires induction of a number of different target genes [28]. As telomere maintenance may contribute to the long-term proliferative potential of tumor cells, telomerase activation may be an essential component of the ability of *myc* to facilitate tumor formation.

Methods

Retroviral plasmids. The following viral plasmids were used for transfection: pBabe-puro [29], MarXII-hygro, mouse c-*myc*/MarXII-hygro (gifts from Dr. P. Sun, CSHL), E6/pBabe-puro, cdc25A/MarXII-hygro. The full length hEST2 cDNA (a gift from Dr. R. Weinberg) was cloned into pBabe-puro vector at the EcoRI and SalI sites.

Cell culture and retroviral-mediated gene transfer. Human mammary epithelial cells (HMEC 184 spiral K) were obtained from Dr. M. Stampher. Normal human diploid fibroblasts (IMR90 and WI38) and human breast cancer cell lines (BT549, T47D and HBL100) were obtained from ATCC. HT1080 cells were a gift from G. Stark (Cleveland Clinic Foundation). The amphotropic packaging line, linX-A, was produced in our laboratory (L. Y. X, D. B. and G. H., unpublished). HMEC were cultured in complete MEGM (Clonetics). Fibroblasts and LinX-A cells were maitained in DMEM (GIBCO) plus 10% fetal bovine serum (FBS; Sigma). BT549, HBL100 and T47D were maintained as directed by the supplier. LinX-A cells were transfected by calcium-phosphate precipitation with a mixture containing 15 μg of retroviral plasmid and 15 μg of sonicated salmon sperm DNA. Transfected cells were incubated at 37°C for 24 hr and then shifted to 30°C for virus production. After 48 hr, the virus was collected, and the virus-containing medium was filtered to remove packaging cells (0.45 μm filter; Millipore). Target cells were infected with virus supernatants supplemented with 4 μg/ml polybrene (Sigma) by

centrifuging for 1 hr at 1000 g and then incubating at 30°C overnight. The infected cells were selected 48 hours after infection using appropriate drugs (hygro*mycin*, G418 or puro*mycin*).

TRAP assays. The TRAP assay was performed essentially as described [1] with some modification. Briefly, extracts were prepared in lysis buffer (10 mM Tris [pH 7.5], 1 mM $MgCl_2$, 1 mM EGTA, 10% Glycerol), and cleared by centrifugation for 30 min at 50,000xg. Lysate corresponding to from 10 to 10^4 cells was used in the assay. Telomeric repeats were synthesized onto an oligonucleotide, TS (5' AATCCGTCGAGCAGAGTT3'), in an extension reaction that proceeded at 30°C for 1 hr. Extension products were amplified by polymerase chain reaction (PCR) in the presence of ^{32}P-dATP using TS in combination with a downstream anchor primer (5' GCGCGGCTAACCCTAACCCTAACC 3'). Five microliters of each reaction was analyzed on a 6% acrylamide / 8 M urea gel.

Northern blotting. Total RNA was isolated from subconfluent cultures using Trizol reagent (GIBCO BRL). Ten micrograms of total RNA was resolved by electrophoresis and transferred to Hybond-N+ membranes according to the manufacturer's instructions. hEST2 was visualized following hybridization with a labelled Stu I fragment of hEST2 as described [20].

Western blotting. Western blotting was performed essentially as described [30]. Cells were washed with cold PBS and lysed in Laemmli loading buffer. Lysates were heated at 95°C for 10 min. Samples were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were incubated either with a c-*myc* rabbit polyclonal antibody (N-262; Santa Crutz) or with a TFIIB rabbit polyclonal antibody (a gift from Dr. B. Tansey). Immune complexes were visualize by secondary incubation with ^{125}I-protein A (ICN).

TRF analysis. Telomeric restriction fragment length was measured precisely as described previously[22].

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

We Claim:

1. A method for increasing the proliferative capacity of cells, comprising contacting the cell a telomerase-activating therapeutic agent.

2. A method for increasing the number of mitotic divisions a cell can undergo, comprising contacting the cell with an agent which increases the level of a telomerase catalytic subunit in the cell, which is selected from the group consisting of (i) an expression construct encoding an EST2 polypeptide or other telomerase activator protein, (ii) an agent which increases or activates expression of an endogenous EST2 gene, (iii) a telomerase activator polypeptide formulated for transcellular uptake, (iv) an agent which inhibits inactivation of endogenous an EST2 protein or *myc* protein, and (v) an agent which derepresses *myc*.

3. The method of claim 2, wherein the EST2 polypeptide is identical or homologous to SEQ ID No. 2.

4. The method of claim 2, wherein the EST2 polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1.

5. The method of claim 2, wherein the expression construct is a vector comprising
   (i) one or more transposition elements for integration of the vector into chromosomal DNA of a eukaryotic host cell;
   (ii) a coding sequence of a telomerase activator; and
   (ii) excision elements for inactivating expression of the coding sequence upon contact with an excision agent.

6. The method of claim 5, wherein vector is a retroviral or lentiviral vector.

7. The method of claim 5 or 6, wherein the excision elements are recombinase recognition sites.

8. The method of claim 7, wherein the recombinase recognition sites are present in the transposition elements such that, upon contacting the cell with the excision agent, all or substantially all of the vector is excised from the chromosome of the cell.

9. The method of claim 2, wherein the agent is an RNA molecule encoding the telomerase activator.

10. The method of claim 2, wherein the agent which inhibits inactivation of an endogenous an EST2 protein or *myc* protein by inhibiting post-translational modification of the protein and/or inhibiting proteolytic degradation of the protein.

11. The method of claim 10, wherein the agent inhibits ubiquitin-mediated degradation of *myc*.

12. The method of claim 2, wherein the agent depresses mad-dependent antagonism of *myc*.

13. The methofd of any of claims 2, 10, 11 or 12, wherein the agent is a small organic molecule.

14. The method of claim 2, wherein the cell is a stem cell or progenitor cells.

15. The method of claim 14, wherein the cell is selected from the group consisting of neuronal, hematopoietic, pancreatic, and hepatic stem and progenitor cells.

16. The method of claim 2, wherein the cell is an epithelial cell.

17. The method of claim 2, wherein the cell is a mesenchymal cell.

18. The method of claim 2, wherein the cell is a chondrocyte or osteocyte.

19. The method of any of claims 1-18, wherein the cell is contacted with the agent in a culture or in *ex vivo* explant.

20. The method of any of claims 1-18, wherein the cell is contacted with the agent *in vivo*.

21. The method of claim 20, wherein the agent is administered to a mammal.

22. The method of claim 21, wherein the mammal is a human.

23. The method of claim 20, wherein the agent is administered as a pharmaceutical preparation.

24. The method of claim 20, wherein the agent is administered as a cosmetic preparation.

25. A pharmaceutical preparation comprising, as an active component, a telomerase-activating therapeutic agent, and a pharmaceutically acceptable excipient

26. A cosemetic preparation comprising, as an active component, a telomerase-activating therapeutic agent, in an amount suitable to promote proliferation of cells of a dermal layer when applied topically, and a pharmaceutically acceptable excipient for topical application.

27. The preparation of claim 25 or 26, wherein the telomerase-activating therapeutic agent is a nucleic acid which encodes a telomerase activating polypeptide

28. The preparation of claim 27, wherien the telomerase activating polypeptide includes an EST2 amino acid sequence, a *myc* amino acid sequence or an E6 amino acid sequence.

28. The preparation of claim 27, wherein the nucleic acid is a vector comprising

(i) one or more transposition elements for integration of the vector into chromosomal DNA of a eukaryotic host cell;

(ii) a coding sequence of a telomerase activator; and

(ii) excision elements for inactivating expression of the coding sequence upon contact with an excision agent.

29. The preparation of claim 28, wherein vector is a retroviral or lentiviral vector.

30. The preparation of claim 28 or 29, wherein the excision elements are recombinase recognition sites.

31. The preparation of claim 30, wherein the recombinase recognition sites are present in the transposition elements such that, upon contacting the cell with the excision agent, all or substantially all of the vector is excised from the chromosome of the cell.

32. The preparation of claim 25 or 26, wherein the telemorase-activating therapeutic agent is an RNA molecule encoding the telomerase activator.

33. The preparation of claim 25 or 26, wherein the teleomerase-activating therapeutic agent inhibits inactivation of an endogenous an EST2 protein or *myc* protein by inhibiting post-translational modification of the protein and/or inhibiting proteolytic degradation of the protein.

34. The preparation of claim 33, wherein the agent inhibits ubiquitin-mediated degradation of *myc*.

35. The preparation of claim 25 or 26, wherein the agent depresses mad-dependent antagonism of *myc*.

36. The preparation of claim 25 or 26, wherein the agent is a small organic molecule.

37. A method for promoting the healing of a wound comprising contacting the wound site on a patient with an a telomerase-activating therapeutic agent, such as which causes ectopic expression of a polypeptide including an EST2 amino acid sequence identical or homologous to SEQ ID No. 2 or a portion thereof, in an amount sufficient to induce cell proliferation.

38. The method of claim 37, wherein the wound site includes epithelial tissue, and the telomerase-activating therapeutic agent promotes proliferation of the epithelial tissue.

39. The method of claim 37, wherein the wound results from surgery, burns, inflammation or irritation.

40. The method claim 37, wherein the agent is applied prophylactically, such as in the form of a cosmetic preparation, to enhance tissue regeneration processes, e.g., of the skin, hair and/or fingernails.

41. The method of claim 37, wherein the wounds is a dermal ulcer.

42. The method of claim 41, wherein the dermal ulcers is a result from venous disease (venous stasis ulcers), excessive pressure (decubitus ulcers) or arterial ulcers.

43. A kit for conjoint administration comprising, (a) the preparation of claim 25 or 26, and (b) a trophic factor.

44. A kit for conjoint administration comprising, (a) the preparation of claim 25 or 26, and (b) a tropic factor.

45. A kit for conjoint administration comprising, (a) the preparation of claim 25 or 26, and (b) a tropic factor.

46. A kit for conjoint administration comprising, (a) the preparation of claim 25 or 26, and (b) a mitogenic agent.

47. The kit of claim 46, wherein the mitogenic agent is a lectins, insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), or a transforming growth factor (TGF).

48. The method of claim 2, wherein the agent is co-administered with a second agent that relieves capping inhibition of EST2 rescue.

49. A kit for conjoint administration comprising, (a) the preparation of claim 25 or 26, and (b) a second agent that relieves capping inhibition of EST2 rescue.

50. The method of claim 48 or the kit of claim 49, wherein the second agent is (a) an oligonucleotide which competes with telomeres for binding of telomere binding proteins, (b) a dominant negative mutant of a telomere binding protein which inhibits formation of inhibitory protein complexes with the telomeric sequences, or (c) an inhibitor of expression of a telomere binding proteins.

51. A method for *ex vivo* therapy comprising

(i) isolating, in cell culture, a population of cells which are to be transplanted to a patient;

(ii) contacting the cells with a telomerase-activating therapeutic agent in an amount sufficeint to increase the number of mitotic divisions the cells can undergo in culture; and

(iii) transplanting the cells into the patient.

52. The method of claim 52, wherein the telomerase-activating therapeutic agent is removed from the cells or inactivated before transplanting the cells into the patient.

SUBSTITUTE SHEET (RULE 26)

```
1                MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPQGNRLVQRGDPAAFRAL  hEST2

51   VAQCLVCVPWDARPPPAAPSFRQVSCLKELVARVLQRLCERGAKNVLAFGFALLDGARGG  hEST2
1    MEVDVDNQADNHGIHSALKTCEEIKEAKTLYS-NIQKVI-RCRNQSQSHYKDLEDIKIFA  p123
1    ---------------------------MKILFE-FIQDKLDIDLQTNSTYKENLKCGHFNG  Est2p

111  PPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRR-VGDDVLVHLLARCALFVLVAPSCAYQ  hEST2
59   QTNIVATP-RDYNEEDFKVIARKEVFSTGLMIE-LIDKCLVELLSS----SDVSDRQKLQ  p123
34   LDEILTT----CFALPNSRKIALPCLPGDLSHKAVIDHCIIYLL---------TGELYNN  Est2p

179  VCGPPLYQLGAATQARP-PPHASGPRRRLGCERAWNHSVREAGVPLGLPAPGARRRGGSA  hEST2
113  CFG---FQLKGNQLAKTHLLTALSTQKQYFFQDEWN-QVR---------AMIGNELFRHL  p123
81   VLT------FGYKIAR---------------NEDVNNSL--------------------F  Est2p

229  SRSLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSPARPAEEATSLEGA  hEST2
160  YTKYLIFQRTSEGTLVQ----FCGNNVFDHLKVNDKFDKK--------QKGGAADMNEPR  p123
100  CHSANVNVTLLKGAAWKMFHSLVGTYAFVDLLINYTVIQFN---GQFFTQIVGNRCNEPH  Est2p

289  LSGTRHSHPSVGRQHHAGPPSTSRPPRPNDTPCPPVYAETKHFLYSSGDK-EQLRPSFLL  hEST2
208  CCSTCKYNVKNEKDHF---LNNINVPN-NNN----MKSRTRIFYCTHFNRNNQFFKKHEF  p123
157  LPPKWVQRSSSSSATAA-QIKWLTEP--V---------TNKQFLHKLNINSSSFFPYSKI  Est2p

348  SSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFLELLGNHAQCPYGY  hEST2
260  VSNKNNISAMDR-AQTIF--TNIFRFNRIRK--KLKDKVIEKIAYMLEKVKDF---NFNY  p123
205  LPSSSSIKKLTDLREAIF-PTNLVKIPQ-----RLKVRINLTLQKLLKRHKRLN---YVS  ESt2p
```

Fig. 1A

```
408  LLKTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRRLVQLLRQHSSPWQVYG-FVR  hEST2
312  YLTKSCPL-----PENNRERKQKIENLINKTREEKS---KYYEELFSYTTDNKCVTQFIN  p123
256  ILNSICP------PLEGTVLD--------------------LSHLSRQSPKERVL-KFII  Est2p

467  ACLRRLVPPGLWGSRHNERRFLRNTKKFISLGKHAKLSLQELTWKMSVRGCAWLRRSPGV  hEST2
364  EFFYNILPKDFLTGR-NRKNFQKKVKKYVELNKHELIHKNLLLEKINTREISWMQVETSA  p123
289  VILQKLLPQEMFGSKKNKGKIIKNLNLLLSLPLNGYLPFDSLLKKLRLKDFRWLFI--SD  Est2p

527  GCVPAAEHRLREEILAKFLHWLMSVYVVELLRSFFYVTETTFQKNRLFFYRKSVWSKLQS  hEST2
423  KHFYYFDHE-NIYVLWKLLRWIFEDLVVSLIRCFFYVTEQQKSYSKTYYYRKNIWDVIMK  p123
347  IWFTKHNFENLNQLAICFISWLFROLIPKIIQTFFYCTEISSTV-TIVYFRHDTWNKLIT  Est2p

                                    motif 1        motif 2
587  IGIRQHLKRVQLRELSEAEVRQHREAR--PALLTSRLRFIPK--PDGLRPI-VNMDY-VV  hEST2
482  MSI-ADLKKETLAEVQEKEVEEWKKS---LGFAPGKLRLIPK--KTTFRPI-MTFN----  p123
486  PFI-VEYFKTYLV---ENNVCRNHNSYTLSNFNHSKMRIIPKKSNNEFRITAIPCRGADE  Est2p

641  GARTFRREKRAERLTSRVKALFSVLNYERARRPGL---LGASVLGLDDIHRAWRTFVLRV  hEST2
531  -KKIVNSDRKTTKLTTNTKLLNSHLMLKTLKNRMFKDPFGFAVFNYDDVMKKYEEFVCKW  p123
462  EEFTIYKENHKNAIOPTOKIL-EYLRNKRPTSFT--KIYSPTQIA-DRIKEFKQRLLKKF  ESt2p

                motif 3
698  RAQDPPPELYFVKVDVTGAYDTIPQDRLTEVI-------------ASIIKPQNTYCVRR  hEST2
590  K-QVGQPKLFFATMDIEKCYDSVNREKLSTFLKTTKLLSSDFWIMTAQILKRKNNIVIDS  p123
518  --NNVLPELYFMKFDVKSCYDSIPRMECMRILK-------------DALKNENGFFVRS  Est2p
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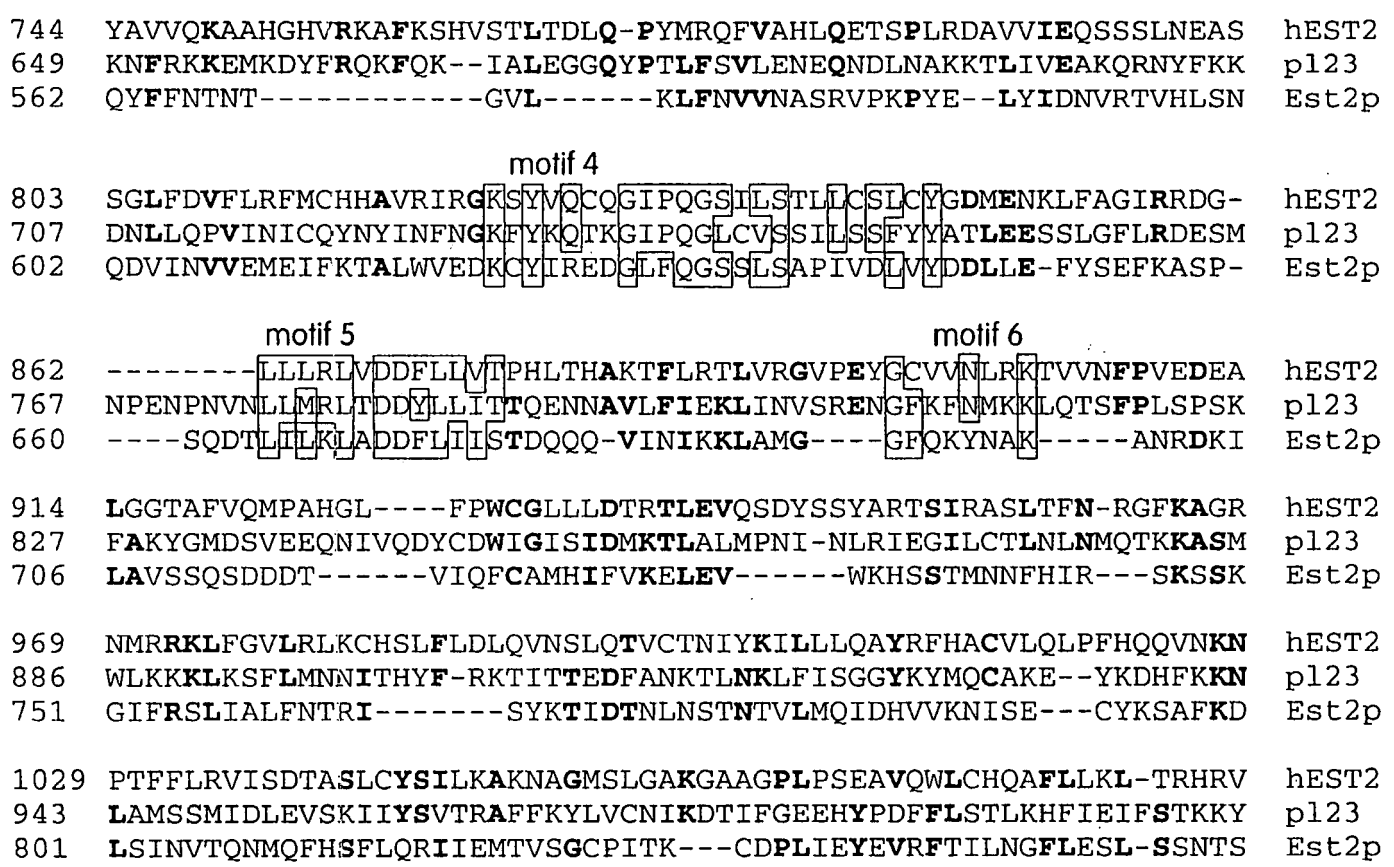
Fig. 1B

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562  QYFFNTNT------------GVL------KLFNVVNASRVPKPYE--LYIDNVRTVHLSN  Est2p

803  SGLFDVFLRFMCHHAVRIRGKSYVQCQGIPQGSILSTLLCSLCYGDMENKLFAGIRRDG-  hEST2
707  DNLLQPVINICQYNYINFNGKFYKQTKGIPQGLCVSSILSSFYYATLEESSLGFLRDESM  p123
602  QDVINVVEMEIFKTALWVEDKCYIREDGLFQGSSLSAPIVDLVYDDLLE-FYSEFKASP-  Est2p

862  --------LLLRLVDDFLLVTPHLTHAKTFLRTLVRGVPEYGCVVNLRKTVVNFPVEDEA  hEST2
767  NPENPNVNLLMRLTDDYLLITTQENNAVLFIEKLINVSRENGFKFNMKKLQTSFPLSPSK  p123
660  ----SQDTLILKLADDFLIISTDQQQ-VINIKKLAMG----GFQKYNAK-----ANRDKI  Est2p

914  LGGTAFVQMPAHGL----FPWCGLLLDTRTLEVQSDYSSYARTSIRASLTFN-RGFKAGR  hEST2
827  FAKYGMDSVEEQNIVQDYCDWIGISIDMKTLALMPNI-NLRIEGILCTLNLNMQTKKASM  p123
706  LAVSSQSDDDT------VIQFCAMHIFVKELEV------WKHSSTMNNFHIR---SKSSK  Est2p

969  NMRRKLFGVLRLKCHSLFLDLQVNSLQTVCTNIYKILLLQAYRFHACVLQLPFHQQVNKN  hEST2
886  WLKKKLKSFLMNNITHYF-RKTITTEDFANKTLNKLFISGGYKYMQCAKE--YKDHFKKN  p123
751  GIFRSLIALFNTRI-------SYKTIDTNLNSTNTVLMQIDHVVKNISE---CYKSAFKD  Est2p

1029 PTFFLRVISDTASLCYSILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLLKL-TRHRV  hEST2
943  LAMSSMIDLEVSKIIYSVTRAFFKYLVCNIKDTIFGEEHYPDFFLSTLKHFIEIFSTKKY  p123
801  LSINVTQNMQFHSFLQRIIEMTVSGCPITK---CDPLIEYEVRFTILNGFLESL-SSNTS  Est2p
```

Fig. 1C

```
hEST2   1088  TYVPLLGSLRTAQTQL-SRKLPGTTLTALEAAANPALPSDFKTILD
p123    1003  IFNRVCMILKAKEAKLKSDQC--QSLIQYDA
Est2p    857  KFKDNIILLRKEIQHLQAYIYIYIHIVN
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Fig. 1D

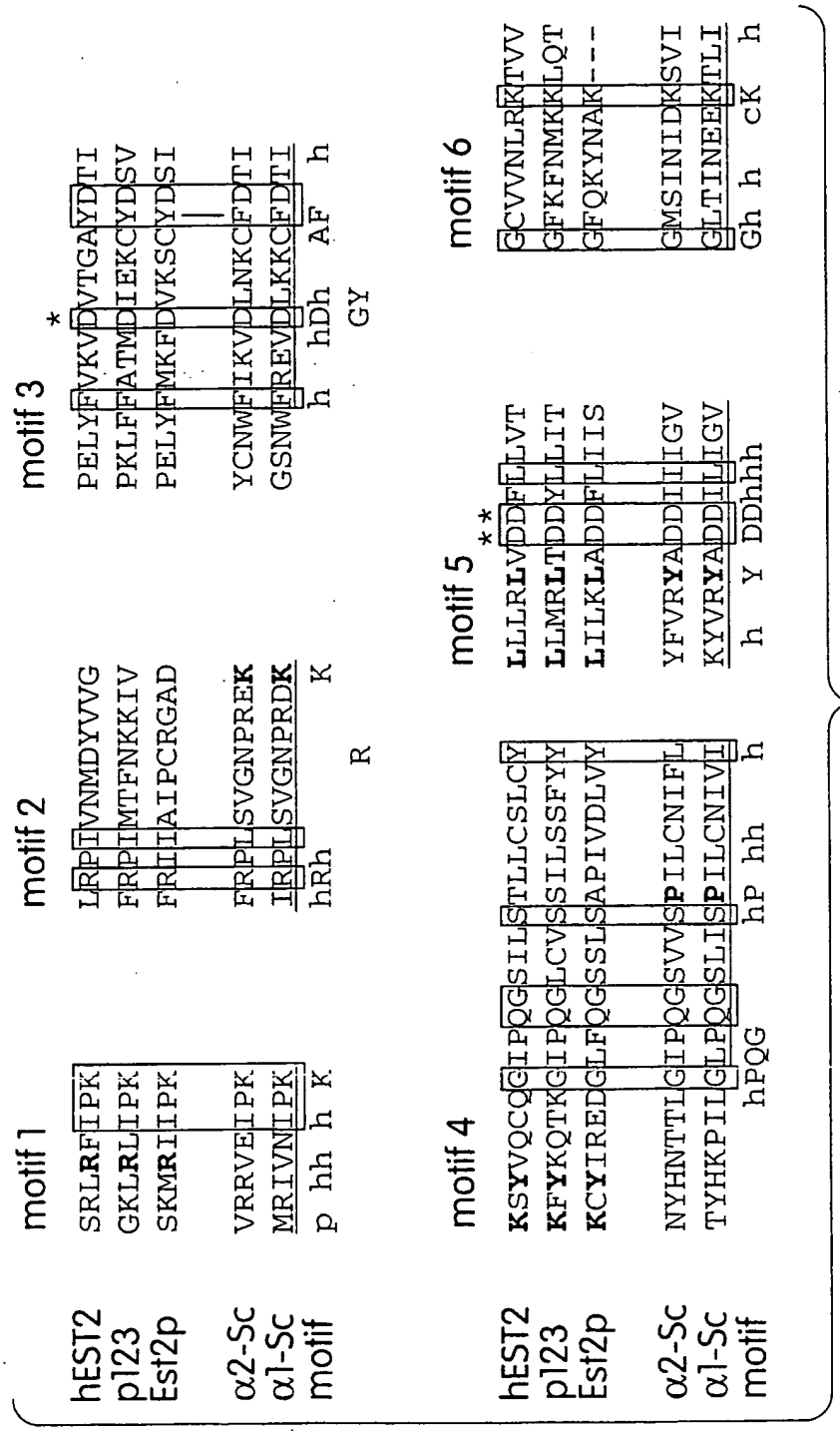
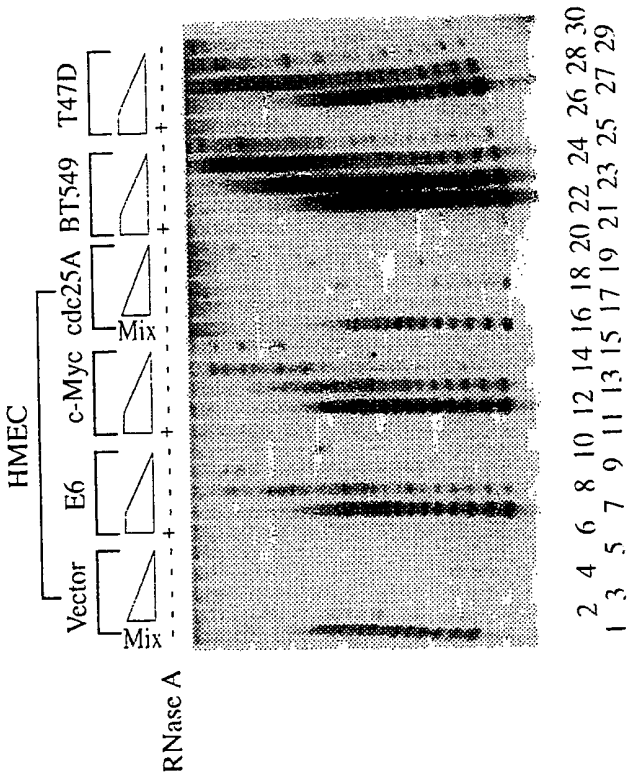

motif 1
motif 2
motif 3
motif 4
motif 5
motif 6
hEST2
p123
Est2p
α2-Sc
α1-Sc
motif

Fig. 2


HMEC
Vector
E6
c-Myc
cdc25A
BT549
T47D
Mix
Mix
RNase A
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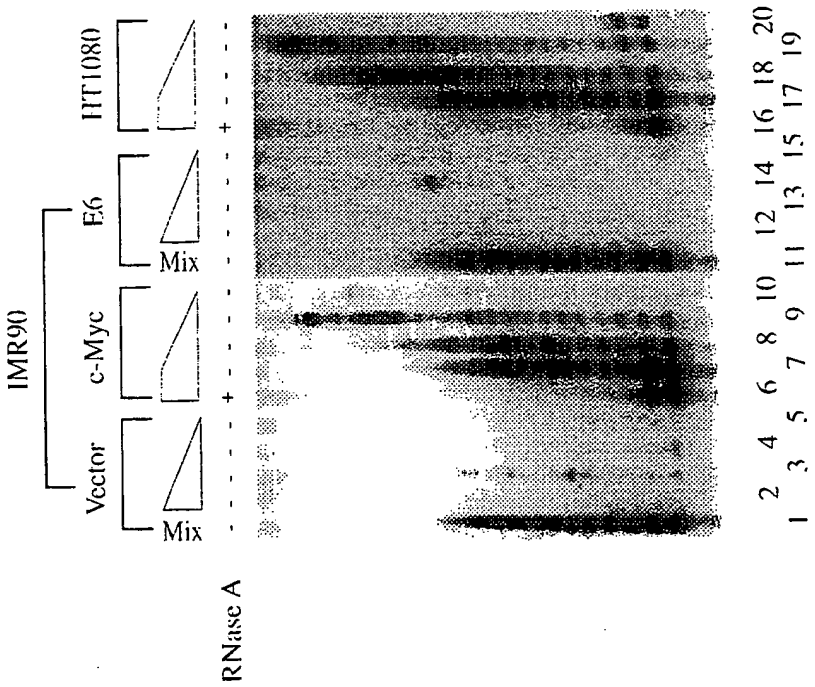
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29

Fig. 3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E6
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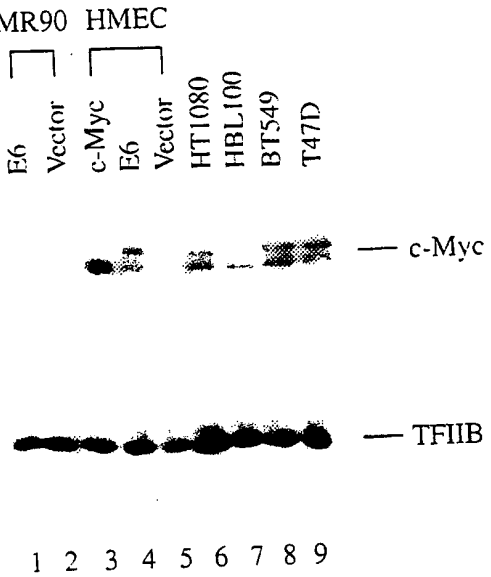
Fig. 4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HMEC
E6
Vector
c-Myc
E6
Vector
HT1080
HBL100
BT549
T47D
c-Myc
TFIIB
1 2 3 4 5 6 7 8 9

Fig. 5A

HMEC
IMR90
Vector
E6
c-Myc
Vector
E6
c-Myc
c-Myc
1 2 3 4 5 6

Fig. 5B

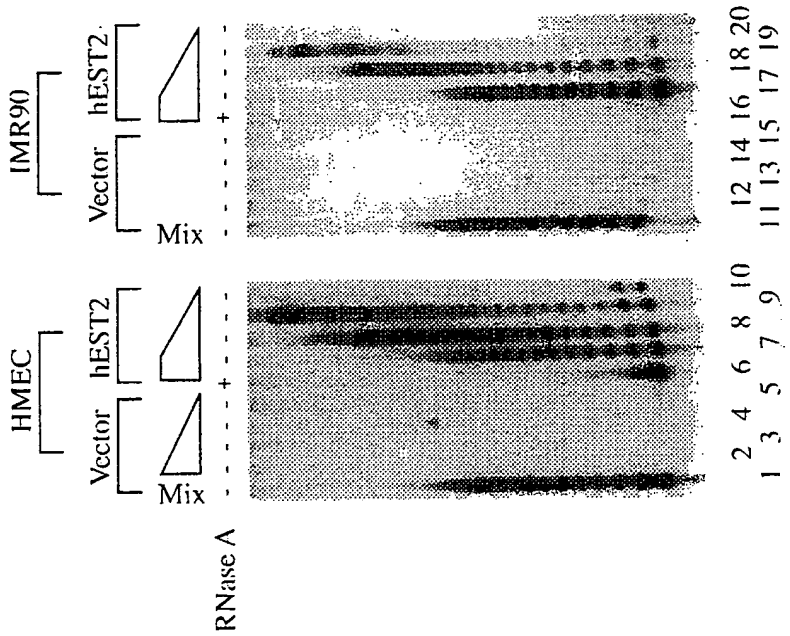


Fig. 6B

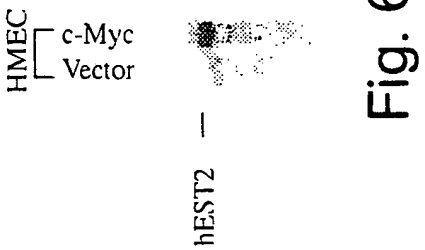


Fig. 6A

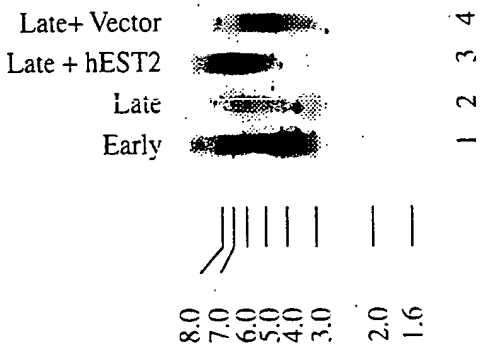
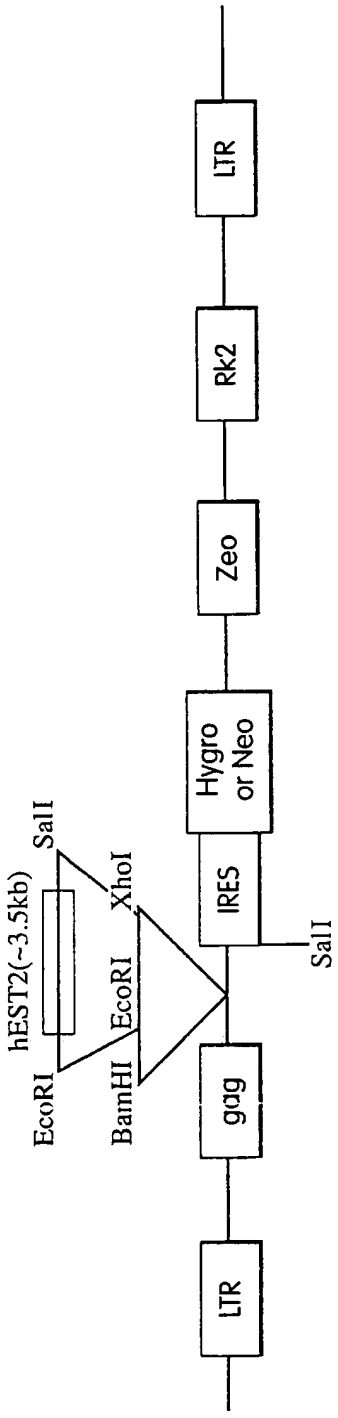


Fig. 6C

Fig. 6D


LTR
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Zeo
Hygro or Neo
IRES
gag
LTR
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XhoI
EcoRI
BamHI
hEST2(~3.5kb)
EcoRI
SalI

Fig. 7

SEQUENCE LISTING

1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: COLD SPRING HARBOR LABORATORY
(B) STREET: ONE BUNGTOWN ROAD
(C) CITY: COLD SPRING HARBOR
(D) STATE: NEW YORK
(E) COUNTRY: US
(F) POSTAL CODE: 11724

(ii) TITLE OF INVENTION: EXTENSION OF CELLULAR LIFESPAN, METHODS AND REAGENTS

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4027 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 57..3452

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CAGGCAGCGT GGTCCTGCTG CGCACGTGGG AAGCCCTGGC CCCGGCCACC CCCGCG          56

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Met Pro Arg Ala Pro Arg Cys Arg Ala Val Arg Ser Leu Leu Arg Ser
 1               5                  10                  15

CAC TAC CGC GAG GTG CTG CCG CTG GCC ACG TTC GTG CGG CGC CTG GGG        152
His Tyr Arg Glu Val Leu Pro Leu Ala Thr Phe Val Arg Arg Leu Gly
            20                  25                  30

CCC CAG GGC TGG CGG CTG GTG CAG CGC GGG GAC CCG GCG GCT TTC CGC        200
Pro Gln Gly Trp Arg Leu Val Gln Arg Gly Asp Pro Ala Ala Phe Arg
        35                  40                  45

GCG CTG GTG GCC CAG TGC CTG GTG TGC GTG CCC TGG GAC GCA CGG CCG        248
Ala Leu Val Ala Gln Cys Leu Val Cys Val Pro Trp Asp Ala Arg Pro
    50                  55                  60

CCC CCC GCC GCC CCC TCC TTC CGC CAG GTG TCC TGC CTG AAG GAG CTG        296
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Pro Pro Ala Ala Pro Ser Phe Arg Gln Val Ser Cys Leu Lys Glu Leu
 65                  70                  75                  80

GTG GCC CGA GTG CTG CAG AGG CTG TGC GAG CGC GGC GCG AAG AAC GTG        344
Val Ala Arg Val Leu Gln Arg Leu Cys Glu Arg Gly Ala Lys Asn Val
                 85                  90                  95

CTG GCC TTC GGC TTC GCG CTG CTG GAC GGG GCC CGC GGG GGC CCC CCC        392
Leu Ala Phe Gly Phe Ala Leu Leu Asp Gly Ala Arg Gly Gly Pro Pro
            100                 105                 110

GAG GCC TTC ACC ACC AGC GTG CGC AGC TAC CTG CCC AAC ACG GTG ACC        440
Glu Ala Phe Thr Thr Ser Val Arg Ser Tyr Leu Pro Asn Thr Val Thr
        115                 120                 125

GAC GCA CTG CGG GGG AGC GGG GCG TGG GGG CTG CTG TTG CGC CGC GTG        488
Asp Ala Leu Arg Gly Ser Gly Ala Trp Gly Leu Leu Leu Arg Arg Val
    130                 135                 140

GGC GAC GAC GTG CTG GTT CAC CTG CTG GCA CGC TGC GCG CTC TTT GTG        536
Gly Asp Asp Val Leu Val His Leu Leu Ala Arg Cys Ala Leu Phe Val
145                 150                 155                 160

CTG GTG GCT CCC AGC TGC GCC TAC CAG GTG TGC GGG CCG CCG CTG TAC        584
Leu Val Ala Pro Ser Cys Ala Tyr Gln Val Cys Gly Pro Pro Leu Tyr
                165                 170                 175

CAG CTC GGC GCT GCC ACT CAG GCC CGG CCC CCG CCA CAC GCT AGT GGA        632
Gln Leu Gly Ala Ala Thr Gln Ala Arg Pro Pro Pro His Ala Ser Gly
            180                 185                 190

CCC CGA AGG CGT CTG GGA TGC GAA CGG GCC TGG AAC CAT AGC GTC AGG        680
Pro Arg Arg Arg Leu Gly Cys Glu Arg Ala Trp Asn His Ser Val Arg
        195                 200                 205

GAG GCC GGG GTC CCC CTG GGC CTG CCA GCC CCG GGT GCG AGG AGG CGC        728
Glu Ala Gly Val Pro Leu Gly Leu Pro Ala Pro Gly Ala Arg Arg Arg
    210                 215                 220

GGG GGC AGT GCC AGC CGA AGT CTG CCG TTG CCC AAG AGG CCC AGG CGT        776
Gly Gly Ser Ala Ser Arg Ser Leu Pro Leu Pro Lys Arg Pro Arg Arg
225                 230                 235                 240

GGC GCT GCC CCT GAG CCG GAG CGG ACG CCC GTT GGG CAG GGG TCC TGG        824
Gly Ala Ala Pro Glu Pro Glu Arg Thr Pro Val Gly Gln Gly Ser Trp
                245                 250                 255

GCC CAC CCG GGC AGG ACG CGT GGA CCG AGT GAC CGT GGT TTC TGT GTG        872
Ala His Pro Gly Arg Thr Arg Gly Pro Ser Asp Arg Gly Phe Cys Val
            260                 265                 270

GTG TCA CCT GCC AGA CCC GCC GAA GAA GCC ACC TCT TTG GAG GGT GCG        920
Val Ser Pro Ala Arg Pro Ala Glu Glu Ala Thr Ser Leu Glu Gly Ala
        275                 280                 285

CTC TCT GGC ACG CGC CAC TCC CAC CCA TCC GTG GGC CGC CAG CAC CAC        968
Leu Ser Gly Thr Arg His Ser His Pro Ser Val Gly Arg Gln His His
    290                 295                 300

GCG GGC CCC CCA TCC ACA TCG CGG CCA CCA CGT CCC TGG GAC ACG CCT       1016
Ala Gly Pro Pro Ser Thr Ser Arg Pro Pro Arg Pro Trp Asp Thr Pro
305                 310                 315                 320

TGT CCC CCG GTG TAC GCC GAG ACC AAG CAC TTC CTC TAC TCC TCA GGC       1064
Cys Pro Pro Val Tyr Ala Glu Thr Lys His Phe Leu Tyr Ser Ser Gly
                325                 330                 335
```

GAC AAG GAG CAG CTG CGG CCC TCC TTC CTA CTC AGC TCT CTG AGG CCC 1112
Asp Lys Glu Gln Leu Arg Pro Ser Phe Leu Leu Ser Ser Leu Arg Pro
340 345 350

AGC CTG ACT GGC GCT CGG AGG CTC GTG GAG ACC ATC TTT CTG GGT TCC 1160
Ser Leu Thr Gly Ala Arg Arg Leu Val Glu Thr Ile Phe Leu Gly Ser
355 360 365

AGG CCC TGG ATG CCA GGG ACT CCC CGC AGG TTG CCC CGC CTG CCC CAG 1208
Arg Pro Trp Met Pro Gly Thr Pro Arg Arg Leu Pro Arg Leu Pro Gln
370 375 380

CGC TAC TGG CAA ATG CGG CCC CTG TTT CTG GAG CTG CTT GGG AAC CAC 1256
Arg Tyr Trp Gln Met Arg Pro Leu Phe Leu Glu Leu Leu Gly Asn His
385 390 395 400

GCG CAG TGC CCC TAC GGG GTG CTC CTC AAG ACG CAC TGC CCG CTG CGA 1304
Ala Gln Cys Pro Tyr Gly Val Leu Leu Lys Thr His Cys Pro Leu Arg
405 410 415

GCT GCG GTC ACC CCA GCA GCC GGT GTC TGT GCC CGG GAG AAG CCC CAG 1352
Ala Ala Val Thr Pro Ala Ala Gly Val Cys Ala Arg Glu Lys Pro Gln
420 425 430

GGC TCT GTG GCG GCC CCC GAG GAG GAG GAC ACA GAC CCC CGT CGC CTG 1400
Gly Ser Val Ala Ala Pro Glu Glu Glu Asp Thr Asp Pro Arg Arg Leu
435 440 445

GTG CAG CTG CTC CGC CAG CAC AGC AGC CCC TGG CAG GTG TAC GGC TTC 1448
Val Gln Leu Leu Arg Gln His Ser Ser Pro Trp Gln Val Tyr Gly Phe
450 455 460

GTG CGG GCC TGC CTG CGC CGG CTG GTG CCC CCA GGC CTC TGG GGC TCC 1496
Val Arg Ala Cys Leu Arg Arg Leu Val Pro Pro Gly Leu Trp Gly Ser
465 470 475 480

AGG CAC AAC GAA CGC CGC TTC CTC AGG AAC ACC AAG AAG TTC ATC TCC 1544
Arg His Asn Glu Arg Arg Phe Leu Arg Asn Thr Lys Lys Phe Ile Ser
485 490 495

CTG GGG AAG CAT GCC AAG CTC TCG CTG CAG GAG CTG ACG TGG AAG ATG 1592
Leu Gly Lys His Ala Lys Leu Ser Leu Gln Glu Leu Thr Trp Lys Met
500 505 510

AGC GTG CGG GGC TGC GCT TGG CTG CGC AGG AGC CCA GGG GTT GGC TGT 1640
Ser Val Arg Gly Cys Ala Trp Leu Arg Arg Ser Pro Gly Val Gly Cys
515 520 525

GTT CCG GCC GCA GAG CAC CGT CTG CGT GAG GAG ATC CTG GCC AAG TTC 1688
Val Pro Ala Ala Glu His Arg Leu Arg Glu Glu Ile Leu Ala Lys Phe
530 535 540

CTG CAC TGG CTG ATG AGT GTG TAC GTC GTC GAG CTG CTC AGG TCT TTC 1736
Leu His Trp Leu Met Ser Val Tyr Val Val Glu Leu Leu Arg Ser Phe
545 550 555 560

TTT TAT GTC ACG GAG ACC ACG TTT CAA AAG AAC AGG CTC TTT TTC TAC 1784
Phe Tyr Val Thr Glu Thr Thr Phe Gln Lys Asn Arg Leu Phe Phe Tyr
565 570 575

CGG AAG AGT GTC TGG AGC AAG TTG CAA AGC ATT GGA ATC AGA CAG CAC 1832
Arg Lys Ser Val Trp Ser Lys Leu Gln Ser Ile Gly Ile Arg Gln His
580 585 590

TTG AAG AGG GTG CAG CTG CGG GAG CTG TCG GAA GCA GAG GTC AGG CAG 1880

```
Leu Lys Arg Val Gln Leu Arg Glu Leu Ser Glu Ala Glu Val Arg Gln
        595                 600                 605

CAT CGG GAA GCC AGG CCC GCC CTG CTG ACG TCC AGA CTC CGC TTC ATC  1928
His Arg Glu Ala Arg Pro Ala Leu Leu Thr Ser Arg Leu Arg Phe Ile
    610                 615                 620

CCC AAG CCT GAC GGG CTG CGG CCG ATT GTG AAC ATG GAC TAC GTC GTG  1976
Pro Lys Pro Asp Gly Leu Arg Pro Ile Val Asn Met Asp Tyr Val Val
625                 630                 635                 640

GGA GCC AGA ACG TTC CGC AGA GAA AAG AGG GCC GAG CGT CTC ACC TCG  2024
Gly Ala Arg Thr Phe Arg Arg Glu Lys Arg Ala Glu Arg Leu Thr Ser
                645                 650                 655

AGG GTG AAG GCA CTG TTC AGC GTG CTC AAC TAC GAG CGG GCG CGG CGC  2072
Arg Val Lys Ala Leu Phe Ser Val Leu Asn Tyr Glu Arg Ala Arg Arg
            660                 665                 670

CCC GGC CTC CTG GGC GCC TCT GTG CTG GGC CTG GAC GAT ATC CAC AGG  2120
Pro Gly Leu Leu Gly Ala Ser Val Leu Gly Leu Asp Asp Ile His Arg
        675                 680                 685

GCC TGG CGC ACC TTC GTG CTG CGT GTG CGG GCC CAG GAC CCG CCG CCT  2168
Ala Trp Arg Thr Phe Val Leu Arg Val Arg Ala Gln Asp Pro Pro Pro
    690                 695                 700

GAG CTG TAC TTT GTC AAG GTG GAT GTG ACG GGC GCG TAC GAC ACC ATC  2216
Glu Leu Tyr Phe Val Lys Val Asp Val Thr Gly Ala Tyr Asp Thr Ile
705                 710                 715                 720

CCC CAG GAC AGG CTC ACG GAG GTC ATC GCC AGC ATC ATC AAA CCC CAG  2264
Pro Gln Asp Arg Leu Thr Glu Val Ile Ala Ser Ile Ile Lys Pro Gln
                725                 730                 735

AAC ACG TAC TGC GTG CGT CGG TAT GCC GTG GTC CAG AAG GCC GCC CAT  2312
Asn Thr Tyr Cys Val Arg Arg Tyr Ala Val Val Gln Lys Ala Ala His
            740                 745                 750

GGG CAC GTC CGC AAG GCC TTC AAG AGC CAC GTC TCT ACC TTG ACA GAC  2360
Gly His Val Arg Lys Ala Phe Lys Ser His Val Ser Thr Leu Thr Asp
        755                 760                 765

CTC CAG CCG TAC ATG CGA CAG TTC GTG GCT CAC CTG CAG GAG ACC AGC  2408
Leu Gln Pro Tyr Met Arg Gln Phe Val Ala His Leu Gln Glu Thr Ser
    770                 775                 780

CCG CTG AGG GAT GCC GTC GTC ATC GAG CAG AGC TCC TCC CTG AAT GAG  2456
Pro Leu Arg Asp Ala Val Val Ile Glu Gln Ser Ser Ser Leu Asn Glu
785                 790                 795                 800

GCC AGC AGT GGC CTC TTC GAC GTC TTC CTA CGC TTC ATG TGC CAC CAC  2504
Ala Ser Ser Gly Leu Phe Asp Val Phe Leu Arg Phe Met Cys His His
                805                 810                 815

GCC GTG CGC ATC AGG GGC AAG TCC TAC GTC CAG TGC CAG GGG ATC CCG  2552
Ala Val Arg Ile Arg Gly Lys Ser Tyr Val Gln Cys Gln Gly Ile Pro
            820                 825                 830

CAG GGC TCC ATC CTC TCC ACG CTG CTC TGC AGC CTG TGC TAC GGC GAC  2600
Gln Gly Ser Ile Leu Ser Thr Leu Leu Cys Ser Leu Cys Tyr Gly Asp
        835                 840                 845

ATG GAG AAC AAG CTG TTT GCG GGG ATT CGG CGG GAC GGG CTG CTC CTG  2648
Met Glu Asn Lys Leu Phe Ala Gly Ile Arg Arg Asp Gly Leu Leu Leu
    850                 855                 860
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CGT TTG GTG GAT GAT TTC TTG TTG GTG ACA CCT CAC CTC ACC CAC GCG 2696
Arg Leu Val Asp Asp Phe Leu Leu Val Thr Pro His Leu Thr His Ala
865 870 875 880

AAA ACC TTC CTC AGG ACC CTG GTC CGA GGT GTC CCT GAG TAT GGC TGC 2744
Lys Thr Phe Leu Arg Thr Leu Val Arg Gly Val Pro Glu Tyr Gly Cys
885 890 895

GTG GTG AAC TTG CGG AAG ACA GTG GTG AAC TTC CCT GTA GAA GAC GAG 2792
Val Val Asn Leu Arg Lys Thr Val Val Asn Phe Pro Val Glu Asp Glu
900 905 910

GCC CTG GGT GGC ACG GCT TTT GTT CAG ATG CCG GCC CAC GGC CTA TTC 2840
Ala Leu Gly Gly Thr Ala Phe Val Gln Met Pro Ala His Gly Leu Phe
915 920 925

CCC TGG TGC GGC CTG CTG CTG GAT ACC CGG ACC CTG GAG GTG CAG AGC 2888
Pro Trp Cys Gly Leu Leu Leu Asp Thr Arg Thr Leu Glu Val Gln Ser
930 935 940

GAC TAC TCC AGC TAT GCC CGG ACC TCC ATC AGA GCC AGT CTC ACC TTC 2936
Asp Tyr Ser Ser Tyr Ala Arg Thr Ser Ile Arg Ala Ser Leu Thr Phe
945 950 955 960

AAC CGC GGC TTC AAG GCT GGG AGG AAC ATG CGT CGC AAA CTC TTT GGG 2984
Asn Arg Gly Phe Lys Ala Gly Arg Asn Met Arg Arg Lys Leu Phe Gly
965 970 975

GTC TTG CGG CTG AAG TGT CAC AGC CTG TTT CTG GAT TTG CAG GTG AAC 3032
Val Leu Arg Leu Lys Cys His Ser Leu Phe Leu Asp Leu Gln Val Asn
980 985 990

AGC CTC CAG ACG GTG TGC ACC AAC ATC TAC AAG ATC CTC CTG CTG CAG 3080
Ser Leu Gln Thr Val Cys Thr Asn Ile Tyr Lys Ile Leu Leu Leu Gln
995 1000 1005

GCG TAC AGG TTT CAC GCA TGT GTG CTG CAG CTC CCA TTT CAT CAG CAA 3128
Ala Tyr Arg Phe His Ala Cys Val Leu Gln Leu Pro Phe His Gln Gln
1010 1015 1020

GTT TGG AAG AAC CCC ACA TTT TTC CTG CGC GTC ATC TCT GAC ACG GCC 3176
Val Trp Lys Asn Pro Thr Phe Phe Leu Arg Val Ile Ser Asp Thr Ala
1025 1030 1035 1040

TCC CTC TGC TAC TCC ATC CTG AAA GCC AAG AAC GCA GGG ATG TCG CTG 3224
Ser Leu Cys Tyr Ser Ile Leu Lys Ala Lys Asn Ala Gly Met Ser Leu
1045 1050 1055

GGG GCC AAG GGC GCC GCC GGC CCT CTG CCC TCC GAG GCC GTG CAG TGG 3272
Gly Ala Lys Gly Ala Ala Gly Pro Leu Pro Ser Glu Ala Val Gln Trp
1060 1065 1070

CTG TGC CAC CAA GCA TTC CTG CTC AAG CTG ACT CGA CAC CGT GTC ACC 3320
Leu Cys His Gln Ala Phe Leu Leu Lys Leu Thr Arg His Arg Val Thr
1075 1080 1085

TAC GTG CCA CTC CTG GGG TCA CTC AGG ACA GCC CAG ACG CAG CTG AGT 3368
Tyr Val Pro Leu Leu Gly Ser Leu Arg Thr Ala Gln Thr Gln Leu Ser
1090 1095 1100

CGG AAG CTC CCG GGG ACG ACG CTG ACT GCC CTG GAG GCC GCA GCC AAC 3416
Arg Lys Leu Pro Gly Thr Thr Leu Thr Ala Leu Glu Ala Ala Ala Asn
1105 1110 1115 1120

CCG GCA CTG CCC TCA GAC TTC AAG ACC ATC CTG GAC TGATGGCCAC 3462

Pro Ala Leu Pro Ser Asp Phe Lys Thr Ile Leu Asp
1125 1130

CCGCCCACAG CCAGGCCGAG AGCAGACACC AGCAGCCCTG TCACGCCGGG CTCTACGTCC 3522

CAGGGAGGGA GGGGCGGCCC ACACCCAGGC CCGCACCGCT GGGAGTCTGA GGCCTGAGTG 3582

AGTGTTTGGC CGAGGCCTGC ATGTCCGGCT GAAGGCTGAG TGTCCGGCTG AGGCCTGAGC 3642

GAGTGTCCAG CCAAGGGCTG AGTGTCCAGC ACACCTGCCG TCTTCACTTC CCCACAGGCT 3702

GGCGCTCGGC TCCACCCCAG GGCCAGCTTT TCCTCACCAG GAGCCCGGCT TCCACTCCCC 3762

ACATAGGAAT AGTCCATCCC CAGATTCGCC ATTGTTCACC CCTCGCCCTG CCCTCCTTTG 3822

CCTTCCACCC CCACCATCCA GGTGGAGACC CTGAGAAGGA CCCTGGGAGC TCTGGGAATT 3882

TGGAGTGACC AAAGGTGTGC CCTGTACACA GGCGAGGACC CTGCACCTGG ATGGGGGTCC 3942

CTGTGGGTCA AATTGGGGGG AGGTGCTGTG GGAGTAAAAT ACTGAATATA TGAGTTTTTC 4002

AGTTTTGAAA AAAAAAAAAA AAAAA 4027

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1132 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Pro Arg Ala Pro Arg Cys Arg Ala Val Arg Ser Leu Leu Arg Ser
1 5 10 15

His Tyr Arg Glu Val Leu Pro Leu Ala Thr Phe Val Arg Arg Leu Gly
20 25 30

Pro Gln Gly Trp Arg Leu Val Gln Arg Gly Asp Pro Ala Ala Phe Arg
35 40 45

Ala Leu Val Ala Gln Cys Leu Val Cys Val Pro Trp Asp Ala Arg Pro
50 55 60

Pro Pro Ala Ala Pro Ser Phe Arg Gln Val Ser Cys Leu Lys Glu Leu
65 70 75 80

Val Ala Arg Val Leu Gln Arg Leu Cys Glu Arg Gly Ala Lys Asn Val
85 90 95

Leu Ala Phe Gly Phe Ala Leu Leu Asp Gly Ala Arg Gly Gly Pro Pro
100 105 110

Glu Ala Phe Thr Thr Ser Val Arg Ser Tyr Leu Pro Asn Thr Val Thr
115 120 125

Asp Ala Leu Arg Gly Ser Gly Ala Trp Gly Leu Leu Leu Arg Arg Val
130 135 140

Gly Asp Asp Val Leu Val His Leu Leu Ala Arg Cys Ala Leu Phe Val
145 150 155 160

Leu Val Ala Pro Ser Cys Ala Tyr Gln Val Cys Gly Pro Pro Leu Tyr

165 170 175

Gln Leu Gly Ala Ala Thr Gln Ala Arg Pro Pro Pro His Ala Ser Gly
180 185 190

Pro Arg Arg Arg Leu Gly Cys Glu Arg Ala Trp Asn His Ser Val Arg
195 200 205

Glu Ala Gly Val Pro Leu Gly Leu Pro Ala Pro Gly Ala Arg Arg Arg
210 215 220

Gly Gly Ser Ala Ser Arg Ser Leu Pro Leu Pro Lys Arg Pro Arg Arg
225 230 235 240

Gly Ala Ala Pro Glu Pro Glu Arg Thr Pro Val Gly Gln Gly Ser Trp
245 250 255

Ala His Pro Gly Arg Thr Arg Gly Pro Ser Asp Arg Gly Phe Cys Val
260 265 270

Val Ser Pro Ala Arg Pro Ala Glu Glu Ala Thr Ser Leu Glu Gly Ala
275 280 285

Leu Ser Gly Thr Arg His Ser His Pro Ser Val Gly Arg Gln His His
290 295 300

Ala Gly Pro Pro Ser Thr Ser Arg Pro Pro Arg Pro Trp Asp Thr Pro
305 310 315 320

Cys Pro Pro Val Tyr Ala Glu Thr Lys His Phe Leu Tyr Ser Ser Gly
325 330 335

Asp Lys Glu Gln Leu Arg Pro Ser Phe Leu Leu Ser Ser Leu Arg Pro
340 345 350

Ser Leu Thr Gly Ala Arg Arg Leu Val Glu Thr Ile Phe Leu Gly Ser
355 360 365

Arg Pro Trp Met Pro Gly Thr Pro Arg Arg Leu Pro Arg Leu Pro Gln
370 375 380

Arg Tyr Trp Gln Met Arg Pro Leu Phe Leu Glu Leu Leu Gly Asn His
385 390 395 400

Ala Gln Cys Pro Tyr Gly Val Leu Leu Lys Thr His Cys Pro Leu Arg
405 410 415

Ala Ala Val Thr Pro Ala Ala Gly Val Cys Ala Arg Glu Lys Pro Gln
420 425 430

Gly Ser Val Ala Ala Pro Glu Glu Glu Asp Thr Asp Pro Arg Arg Leu
435 440 445

Val Gln Leu Leu Arg Gln His Ser Ser Pro Trp Gln Val Tyr Gly Phe
450 455 460

Val Arg Ala Cys Leu Arg Arg Leu Val Pro Pro Gly Leu Trp Gly Ser
465 470 475 480

Arg His Asn Glu Arg Arg Phe Leu Arg Asn Thr Lys Lys Phe Ile Ser
485 490 495

Leu Gly Lys His Ala Lys Leu Ser Leu Gln Glu Leu Thr Trp Lys Met
500 505 510

Ser Val Arg Gly Cys Ala Trp Leu Arg Arg Ser Pro Gly Val Gly Cys

515 520 525

Val Pro Ala Ala Glu His Arg Leu Arg Glu Glu Ile Leu Ala Lys Phe
530 535 540

Leu His Trp Leu Met Ser Val Tyr Val Val Glu Leu Leu Arg Ser Phe
545 550 555 560

Phe Tyr Val Thr Glu Thr Thr Phe Gln Lys Asn Arg Leu Phe Phe Tyr
565 570 575

Arg Lys Ser Val Trp Ser Lys Leu Gln Ser Ile Gly Ile Arg Gln His
580 585 590

Leu Lys Arg Val Gln Leu Arg Glu Leu Ser Glu Ala Glu Val Arg Gln
595 600 605

His Arg Glu Ala Arg Pro Ala Leu Leu Thr Ser Arg Leu Arg Phe Ile
610 615 620

Pro Lys Pro Asp Gly Leu Arg Pro Ile Val Asn Met Asp Tyr Val Val
625 630 635 640

Gly Ala Arg Thr Phe Arg Arg Glu Lys Arg Ala Glu Arg Leu Thr Ser
645 650 655

Arg Val Lys Ala Leu Phe Ser Val Leu Asn Tyr Glu Arg Ala Arg Arg
660 665 670

Pro Gly Leu Leu Gly Ala Ser Val Leu Gly Leu Asp Asp Ile His Arg
675 680 685

Ala Trp Arg Thr Phe Val Leu Arg Val Arg Ala Gln Asp Pro Pro Pro
690 695 700

Glu Leu Tyr Phe Val Lys Val Asp Val Thr Gly Ala Tyr Asp Thr Ile
705 710 715 720

Pro Gln Asp Arg Leu Thr Glu Val Ile Ala Ser Ile Ile Lys Pro Gln
725 730 735

Asn Thr Tyr Cys Val Arg Arg Tyr Ala Val Val Gln Lys Ala Ala His
740 745 750

Gly His Val Arg Lys Ala Phe Lys Ser His Val Ser Thr Leu Thr Asp
755 760 765

Leu Gln Pro Tyr Met Arg Gln Phe Val Ala His Leu Gln Glu Thr Ser
770 775 780

Pro Leu Arg Asp Ala Val Val Ile Glu Gln Ser Ser Ser Leu Asn Glu
785 790 795 800

Ala Ser Ser Gly Leu Phe Asp Val Phe Leu Arg Phe Met Cys His His
805 810 815

Ala Val Arg Ile Arg Gly Lys Ser Tyr Val Gln Cys Gln Gly Ile Pro
820 825 830

Gln Gly Ser Ile Leu Ser Thr Leu Leu Cys Ser Leu Cys Tyr Gly Asp
835 840 845

Met Glu Asn Lys Leu Phe Ala Gly Ile Arg Arg Asp Gly Leu Leu Leu
850 855 860

Arg Leu Val Asp Asp Phe Leu Leu Val Thr Pro His Leu Thr His Ala

865 870 875 880

Lys Thr Phe Leu Arg Thr Leu Val Arg Gly Val Pro Glu Tyr Gly Cys
885 890 895

Val Val Asn Leu Arg Lys Thr Val Val Asn Phe Pro Val Glu Asp Glu
900 905 910

Ala Leu Gly Gly Thr Ala Phe Val Gln Met Pro Ala His Gly Leu Phe
915 920 925

Pro Trp Cys Gly Leu Leu Leu Asp Thr Arg Thr Leu Glu Val Gln Ser
930 935 940

Asp Tyr Ser Ser Tyr Ala Arg Thr Ser Ile Arg Ala Ser Leu Thr Phe
945 950 955 960

Asn Arg Gly Phe Lys Ala Gly Arg Asn Met Arg Arg Lys Leu Phe Gly
965 970 975

Val Leu Arg Leu Lys Cys His Ser Leu Phe Leu Asp Leu Gln Val Asn
980 985 990

Ser Leu Gln Thr Val Cys Thr Asn Ile Tyr Lys Ile Leu Leu Leu Gln
995 1000 1005

Ala Tyr Arg Phe His Ala Cys Val Leu Gln Leu Pro Phe His Gln Gln
1010 1015 1020

Val Trp Lys Asn Pro Thr Phe Phe Leu Arg Val Ile Ser Asp Thr Ala
1025 1030 1035 1040

Ser Leu Cys Tyr Ser Ile Leu Lys Ala Lys Asn Ala Gly Met Ser Leu
1045 1050 1055

Gly Ala Lys Gly Ala Ala Gly Pro Leu Pro Ser Glu Ala Val Gln Trp
1060 1065 1070

Leu Cys His Gln Ala Phe Leu Leu Lys Leu Thr Arg His Arg Val Thr
1075 1080 1085

Tyr Val Pro Leu Leu Gly Ser Leu Arg Thr Ala Gln Thr Gln Leu Ser
1090 1095 1100

Arg Lys Leu Pro Gly Thr Thr Leu Thr Ala Leu Glu Ala Ala Ala Asn
1105 1110 1115 1120

Pro Ala Leu Pro Ser Asp Phe Lys Thr Ile Leu Asp
1125 1130

INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/US 99/00682

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/17 C12N9/12 A61K7/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 40868 A (COLD SPRING HARBOR LAB ;GREIDER CAROL (US); AUTEXIER CHANTAL (US)) 19 December 1996 see page 2, line 21 - page 3, line 14; claims 13,14,17 see page 5, line 1 - line 12 see page 21, line 12 - line 27 ---	1-4,9
X	K. HIYAMA ET AL.: "ACTIVATION OF TELOMERASE IN HUMAN LYMPHOCYTES AND HEMATOPOIETIC PROGENITOR CELLS" J. IMMUNOLOGY, vol. 155, no. 8, 1995, pages 3711-3715, XP002107651 see the whole document --- -/--	1,2, 13-16,19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 June 1999	12/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Charles, D

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INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/US 99/00682

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	AUTEXIER C ET AL: "RECONSTITUTION OF HUMAN TELOMERASE ACTIVITY AND IDENTIFICATION OF AMINIMAL FUNCTIONAL REGION OF THE HUMAN TELOMERASE RNA" EMBO JOURNAL, vol. 15, no. 21, 1 November 1996, pages 5928-5935, XP002056802 see the whole document	1,2,9,13
X	S.L. WEINRICH ET AL.: "RECONSTITUTION OF HUMAN TELOMERASE WITH THE TEMPLATE RNA COMPONENT HTR AND THE CATALYTIC PROTEIN SUBUBIT HTRT" NATURE GENETICS, vol. 17, 1997, pages 498-502, XP002107652 cited in the application see the whole document	1,2,9,13
X	WO 95 13382 A (GERON CORP ;UNIV CALIFORNIA (US); UNIV TEXAS (US)) 18 May 1995 see page 18, line 10 - page 20, line 13; claims 3,21 see page 23, line 31 - page 24, line 27 see page 162, line 16 - page 165, line 11	1,2
X,P	US 5 830 644 A (WRIGHT WOODRING E ET AL) 3 November 1998 cited in the application see column 13, line 33 - column 14, line 34 see column 30, line 1 - line 8 see column 30, line 32 - line 56 see column 90, line 41 - column 91, line 26	1,2
X,P	H. VAZIRI AND S. BENCHIMOL: "RECONSTITUTION OF TELOMERASE ACTIVITY IN NORMAL HUMAN CELLS LEADS TO ELONGATION OF TELOMERES AND EXTENDED REPLICATIVE LIFE SPAN" CURRENT BIOLOGY, vol. 8, no. 5, 26 February 1998, pages 279-282, XP002107653 see the whole document	1-4,10, 13,19
X,P	J. WANG ET AL.: "MYC ACTIVATES TELOMERASE" GENES & DEVELOPMENT, vol. 12, 1998, pages 1769-1774, XP002107654 cited in the application see the whole document	1,2,10, 13

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INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 99/00682

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	K. FUJIMOTO AND M. TAKAHASHI: "TELOMERASE ACTIVITY IN HUMAN LEUKEMIC CELL LINES IS INHIBITED BY ANTISENSE PENTADECADEOXYNUCLEOTIDES TARGETED AGAINST C-MYC MRNA". BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 241, 1997, pages 775-781, XP002107655 see the whole document -----	1,2,10, 12,14,15

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/US 99/00682

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 20-24, 37-42, 51, 52 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Interi nal Application No
PCT/US 99/00682

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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